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MSc in Medical Biotechnologies

**TERTIARY LYMPHOID TISSUE in COLORECTAL CANCER:
a KEY PLAYER
in the TUMOUR IMMUNE MICROENVIRONMENT**

For the Degree of

Doctor of Philosophy

The Open University, UK

Clinical and Research Institute Humanitas, Rozzano, Milan, Italy

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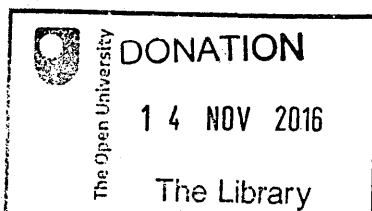
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Abstract

Tumour infiltrating lymphocytes influence colorectal cancer (CRC) progression. However, lymphocyte infiltration comes in different flavours and evidence has been provided that the spatial distribution of immune cells within the tumour tissue is an important immunological feature. The aim of this thesis was to investigate how the dual localization of tumour infiltrating lymphocytes (TILs) can affect their function in the tumour microenvironment. The project started with the analysis of the CD3 compartment, as CD3+ T cell infiltration (CD3-TILs) is a recognized positive prognostic factor for CRC patients. Results here presented show that CD3+ tumour-infiltrating lymphocytes are present both interspersed in the tumour tissue or scattered throughout the stroma (CD3-TILs) and also aggregated in lymphoid structures showing features of tertiary lymphoid tissue (CD3-TLT). Tumour-associated TLT had a peculiar compartmentalization, with CD3+ T cells and CD20+ B lymphocytes holding complementary positions and with distinct types of dendritic cell populations among them. The presence of HEVs (High Endothelial Venules) suggests a role for TLT in T cell recruitment at the tumour site. To test this hypothesis in human cancer, I performed a whole tissue analysis of the CD3+ infiltrate on CRC sections and found a positive correlation between CD3-TIL and CD3-TLT densities. I further confirmed the hypothesis *in vivo* in a murine model of colitis-associated cancer (AOM/DSS). AOM/DSS treated mice had expanded TLT compared to control mice. Intravenously injected GFP+ splenocytes localised in TLT of tumour-bearing mice more than in control mice. I then investigated the clinical significance of CD3-TLT in relationship with CD3-TILs in a cohort of 351 CRC patients. In patients with node-negative (without lymph node metastasis, stage II) CRC, a high density of CD3-TLT and CD3-TILs associated to a better prognosis, while in patients with node-positive (presence of lymph node metastasis, stage III) CRC, TLT and TIL density were irrelevant in predicting patient prognosis, thus behaving as biomarkers only for early stage CRC patients.

In the second part of my thesis, I analysed the distribution of B cells in colorectal cancer and their possible contribution to disease progression. Despite still



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controversial, increasing evidence that B cells play a role in cancer progression has been provided, bringing up the hypothesis that also B-cell responses should be considered as targets of immunotherapeutic approaches. Similarly to CD3+ cells, I showed that, both in human and in preclinical models of CRC, B cells display a dual geographical distribution, either within tertiary lymphoid tissue (CD20-TLT) or dispersed at the tumour invasive margin (CD20-TILs). Therefore, I evaluated the role of B cells according to their localization in the microenvironment. I found that CD20-TLT associated to better prognosis, while CD20-TILs did not. Interestingly, CD20-TLT correlated with CD20-TILs only among patients who experienced cancer recurrence. This result suggests that, when located within a lymphoid site, B cells might have a protective anti-tumour function, participating in an anti-tumour immune response. Conversely, the distribution of B cells scattered in the microenvironment is likely to reflect a non-specific pro-tumour inflammatory reaction. To confirm the hypothesis in vivo and attempt to dissect the dual function of B cells, I took advantage of two CRC preclinical models in which B cells present a distinct geographical distribution in the tumour microenvironment. In the first model, B cells mainly localise within TLT, while in the second one B cells diffusely infiltrate the mucosa, without forming aggregates. I found that in a model in which B cells localize primarily within TLT, the genetic deficiency of B cells significantly increased tumour formation, suggesting that B cells within TLT might exert an important anti-tumour function. In contrast, in a model in which B cells only localize within the tissue, genetic deficiency of B cells reduces tumour growth, suggesting that infiltrating B-TILs might have a pro-tumour role.

Therefore, the occurrence of TLT is associated with lymphocyte infiltration in CRC, contributing to recruitment of CD3-TILs. TLT and TILs work together to set up an anti-tumour immune response in patients with low-risk early-stage colorectal cancer. Thus, TLT represents a novel prognostic biomarker for CRC. As to the B cell compartment, their differential distribution in the tumour site corresponds to distinct prognostic functions. This evidence suggests that the design of novel immunotherapeutic drugs depleting B cells should take into account their ability to selectively targeting CD20-TILs but not C20-TLT.

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INTRODUCTION

COLORECTAL CANCER

Colorectal cancer

Epidemiology

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and one of the leading causes of cancer mortality in the world. It accounts for over 9% of all cancer incidence [1, 2]. The World Health Organization (WHO) evaluates that approximately one million new cases occur worldwide every year [3]. CRC corresponds to the 9.4% of all incident cancers in men and 10.1% in women. It is not uniformly distributed throughout the world [1, 4], as it is more frequent in industrialized than developing countries. Therefore, every population has its own incidence rate of colorectal cancer, which changes overtime. Differently from Northern and Western Europe, the incidence of CRC in the United States is dwindling, a trend that is attributed to screening programs that may have improved the detection of precancerous lesions [5]. In all the other places, however, the incidence is increasing rapidly, in particular in those countries where the transition from a low-income to a high-income economy has recently taken place, such as Japan, Singapore and Eastern European countries [1, 6]. Worldwide differences in the outcome are related to diet, genetic factors, population life span, socio-economic factors, access to therapies and quality of the latest [3].

Risk factors and causes

The possibility of developing colorectal cancer rises gradually from the age of 40, increasing rapidly from the age of 50. More than 90% of colorectal cancer patients are older than 50 years old [4].

Colorectal cancer develops from pre-neoplastic lesions, which are polyps of the colorectum, also known as tubular or villous adenomas [5]. Approximately 95% of sporadic CRC derives from these adenomas [4]. Therefore, adenomas increase the risk of an individual to develop cancer [7]. Adenomas need 5-10 years prior to

malignant transformation; thus, early detection and removal of adenomas reduces cancer risk [8].

Risk factors can be divided in modifiable, usually environmental agents such as diet, sedentary life-style, obesity and smoking, and non-modifiable. The latest include age and hereditary factors, both uncontrollable by an individual. Among others, persistent inflammation of the intestine is emerging as an important risk factor. Inflammatory Bowel Disease (IBD) is a term that includes Ulcerative Colitis and Crohn's disease. In the first one, the lining of the large intestine is the target of chronic inflammation; in the second one, inflammation takes place in the lining and in the wall of the large and/or small intestine. Both these conditions predispose to development of colorectal cancer. The relative risk of CRC in patients with IBD has been estimated between 4- to 20- fold [6].

In approximately 75% of patients, CRC is a sporadic disease [9], where genetic and environmental factors (modifiable factors) are important determinants of the disease (**Table 1**). In these cases, patients don't have a family history of CRC or a predisposing disease. The remaining 25% of patients presents a family history of CRC or adenomatous polyps that suggests a hereditary contribution, common exposure to environmental factors among family members, or a combination of both [3]. In only the 5-6% of all CRC cases, genetic mutations have been associated to inherited cancer risk and a defined hereditary cancer syndrome develops [6]. There are two main syndromes: hereditary non polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) [10]. Genetic mutations responsible for these inherited forms of CRC have been identified. HNPCC patients have mutations in genes involved in the DNA repair pathway, MLH1 (human MutL homolog 1) and MSH2 (human MutS homolog 2) genes. FAP syndrome is due to mutations in the tumour suppressor gene APC (Adenomatous Polyposis Coli). HNPCC accounts for 2 to 6% of colorectal cancer; FAP accounts for less than 1% [9].

Colorectal cancer development is due to the accumulation of genetic alterations (gene mutations, gene amplification, and so on) and epigenetic alterations (aberrant DNA methylation, chromatin modifications, and so on). The earliest steps in the tumorigenesis include genomic instability and, as its consequence, gene alterations. Both events contribute to the transformation from normal colonic epithelium to colon adenocarcinoma, combining alterations in tumour suppressor genes and oncogenes that facilitate cell transformation and tumour progression.

There are two major pathways involved in CRC: the chromosomal instability pathway (CIN) and the microsatellite instability pathway (MSI). Recently, it has been demonstrated that there are other routes, including the serrated and epigenetic pathway. Moreover, all these pathways may establish a cross-talk, highlighting the variety of genes involved in CRC development.

Risk factors and causes

Sporadic colorectal cancer (88-94%)

Older age

Male sex

Cholecystectomy

Ureterocolic anastomosis

Hormonal factors: nulliparity, late age at first pregnancy, early menopause

Environmental factors

Diet rich in meat and fat, and poor in fibre, folate and calcium

Sedentary lifestyle

Obesity

Diabetes mellitus

Smoking

Previous irradiation

Occupational hazards (eg, asbestos exposure)

High alcohol intake

Personal history of sporadic tumours

History of colorectal polyps

History of colorectal cancer

History of small bowel, endometrial, breast or ovarian cancer

Familial colorectal cancer (20%)

First or second degree relatives with this cancer, criteria for hereditary colorectal cancer not fulfilled:

- One affected first-degree relative increases risk 2-3 fold
- Two or more affected first-degree relatives increase risk 4-25 fold
- Index case <45 years increases risk 3-9 fold
- Familial history of colorectal adenoma increases risk 2-fold

Colorectal cancer in inflammatory bowel disease (1-2%)

Ulcerative colitis

Chron's disease

Hereditary colorectal cancer (5-10%)

Polyposis-syndromes: familial adenomatous polyposis (FAP), Gardner's syndrome,

Turcot's syndrome, attenuated adenomatous polyposis coli, flat adenoma syndrome

Hereditary non-polyposis colorectal cancer (HNPCC)

Hamartomatous polyposis syndromes

Table 1. Summary of CRC risk factors and causes. Adapted from Weitz, 2005 [3]

AJCC (TNM) Staging System of colorectal cancer

As mentioned above, a significant heterogeneity is present in the history of individual colorectal tumours. However, based on a specific staging system, broad generalizations can be made concerning the diagnosis and treatment approach of CRC [11]. The AJCC/UICC TNM classification is the most common classification system for the staging and prognostication of cancer; it summarizes data on tumour burden (T), presence of cancer cells in draining and regional LN (N) and evidence of metastases (M). However, it has emerged that patients within the same TNM stage may have a range of clinical outcomes, suggesting that this system should be improved [12].

According to the AJCC (TNM) system, CRC ranges from Stage I to stage IV. Stage I includes primary tumours that invade the sub mucosa (T1) and those invading the muscularis propria (T2), without regional lymph node metastasis (N0) and distant metastasis (M0). Stage II consists of stage IIA, including tumours invading through the muscularis propria into sub serosa (T3), and stage IIB, including tumours that directly invade other organs or structures (T4). Stage II does not include nodal or distant metastasis. Stage III consists of stage IIIA, including T1 and T2 with nodal metastasis, and stage IIIB, including T3 and T4 with nodal metastasis, stage IIIC, including only nodal metastasis with any T. Stage IV shows distant metastasis without any primary tumours or any nodal metastasis. Another factor that can affect the survival expectation is cancer grade. **Table 2** summarizes the characteristics of colorectal cancer staging in this system.

T – Primary tumour			
TX	Primary tumour cannot be assessed		
T0	No evidence of primary tumour		
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria		
T1	Tumour invades submucosa		
T2	Tumour invades muscularis propria		
T3	Tumour invades through the muscularis propria into subserosa or into non-peritonealised pericolic or perirectal tissues		
T4	Tumour directly invades other organs or structures and/or perforates visceral peritoneum		
N – Regional lymph node			
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Metastasis in 1 to 3 regional lymph nodes		
N2	Metastasis in 4 or more regional lymph nodes		
M – Distant metastasis			
MX	Distant metastasis cannot be assessed		
M0	No distant metastasis		
M1	Distant metastasis		
Stage	T	N	M
Stage I	T1, T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4	N0	M0
Stage IIIA	T1, T2	N1	M0
Stage IIIB	T3, T4	N1	M0
Stage IIIC	Any T	N2	M0
Stage IV	Any T	Any N	M1

Table 2. TNM staging system for colorectal cancer.

Tumour staging has been by far the most important prognostic predictor of clinical outcome for patients with CRC [13]. Colorectal cancer survival is strictly related to the stage of the disease at diagnosis, and usually ranges from a 90% 5-year survival rate for cancers detected at localized stage; 70% for regional; to 10% for patients diagnosed for distant metastatic cancer [4]. In general, the earlier the stage at diagnosis, the higher the chance of survival [3]. Mortality related to colorectal cancer is nearly half that of the incidence [3].

Predictive/prognostic markers and therapeutic treatments

Understanding the molecular basis of colorectal cancer has provided tools for genetic testing for high-risk familial forms of the disease. Moreover, drug therapies are selectively allocated to patients thanks to predictive markers, which guide the clinicians, by providing an estimation on how the individual will respond to that therapy. In addition, molecular diagnosis allows a non-invasive detection of early cancers.

Studies aimed at deepen in the molecular aspects of CRC pathogenesis identified biological pathways that may help the development of new therapeutic agents [14]. Until now, the only molecule targeted by clinical therapies is the epidermal growth factor receptor (EGFR) [15-18]. However, several genomic markers are efficient tools for prognosis. For instance, germ-line mutations in tumour-suppressor genes, such as APC, MLH1, MSH2, indicate a high-risk of developing colorectal cancer and are also associated to CRC [19-22].

Other genetic alterations can be considered as prognostic markers. For instance, loss of heterozygosis at chromosome 18q is associated with poor survival in stage II and stage III CRC [21]. MMR deficiency in sporadic CRC usually is associated with a favourable prognosis and a lower chance to develop distant metastasis [23].

Screening programs, including faecal occult-blood test, flexible sigmoidoscopy, double-contrast barium enema and colonoscopy, have considerably reduced CRC mortality [24]. Colonoscopy is the gold standard for diagnosis of CRC, together with abdominal ultrasound and chest radiography [3]. For patient categories with a personal or familial history of colorectal adenomas or colorectal cancer (FAP, HNPCC or IBD) specific surveillance guidelines exist [25].

For early-stage CRC that have not spread to distant sites surgical intervention is the first line therapy. Up to date, surgical techniques have been improved a lot [26]. Chemotherapy may also be used after surgery (adjuvant chemotherapy) if cancer has a higher risk of recurrence. For stage III tumours,

where cancer has spread to nearby lymph nodes, the standard treatment includes surgery followed by adjuvant chemotherapy. For metastatic patients (Stage IV), in which cancer has spread from the colon to distant organs and tissues, chemotherapy is the first line care: FOLFOX (5-Fluorouracil (5-FU), leucovorin, and oxaliplatin) or FOLFIRI (5-FU, leucovorin, and irinotecan) are the most commonly used [27].

Immunotherapy includes therapeutic strategies that harness the immune system to eradicate tumours. CRC, as many other tumours, exerts multiple strategies to suppress and evade the immune system, thus representing a clinical setting in which immunotherapeutic strategies could provide clinical benefit. Among other immunotherapeutic strategies, monoclonal antibodies (mAbs) have shown their clinical effectiveness for decades. Antibodies such as Bevacizumab, directed against VEGF, [28, 29] and cetuximab or panitumumab, both directed against EGFR, have been approved and are currently used for the treatment of CRC in the USA. Many clinical trials are currently testing other mAbs targeting pathways that should induce tumour death, for instance by disruption of vital signalling pathways. Another class of antibodies includes the so called “checkpoint inhibitors”, mAbs that target the inhibitory immune receptors CTLA-4, programmed cell death-1 (PD-1), and PD-1 ligand (PD-L1). In CRC, preliminary studies have shown the limited efficacy of these kind of antibodies, when used as single agents [30, 31]. The poor efficacy of current immunotherapies in CRC revealed the relatively resistant nature of CRC to immune-based strategies. However, combination of conventional chemotherapy or radiation with immunotherapy might be effective in the clinic [32].

Due to the heterogeneity of CRC, patient response to therapies is very inconstant, being the main impediment for the optimization of therapeutic strategies. In order to help the development of new therapeutic treatments, an important aim is to identify biomarkers to predict treatment response [33]. Among emerging biomarkers of prognosis and response to treatment, immune mediators appear to occupy a key position, encouraging the research of immune based therapeutic approaches [34, 35].

The IMMUNE INFILTRATE in COLORECTAL CANCER

Cancer and inflammation

Cancer progression is a multi-step process, which involves several events, including, among the earliest, genomic instability and epigenetic alterations. As a consequence of these events, tumour cells acquire specific features that have been summarized by Hanahan and Weinberg in the six hallmarks of cancer [36]. The first signature of malignant cells highlight intrinsic characteristics of these cells, such as sustaining proliferative signalling or resisting cell death, regardless of what happens in the neighbourhood. Lately, tumour microenvironment has received more attention and its role in the evolution of the disease has been recognized of primary importance. Tumorigenic mechanisms that depend on the interaction between malignant cells and the tumour microenvironment have been included in the emerging hallmarks [37, 38]. Among others, the role of inflammatory mediators in cancer has been recognized, so that inflammation has been integrated as the seventh hallmark of cancer (**Figure 1**).

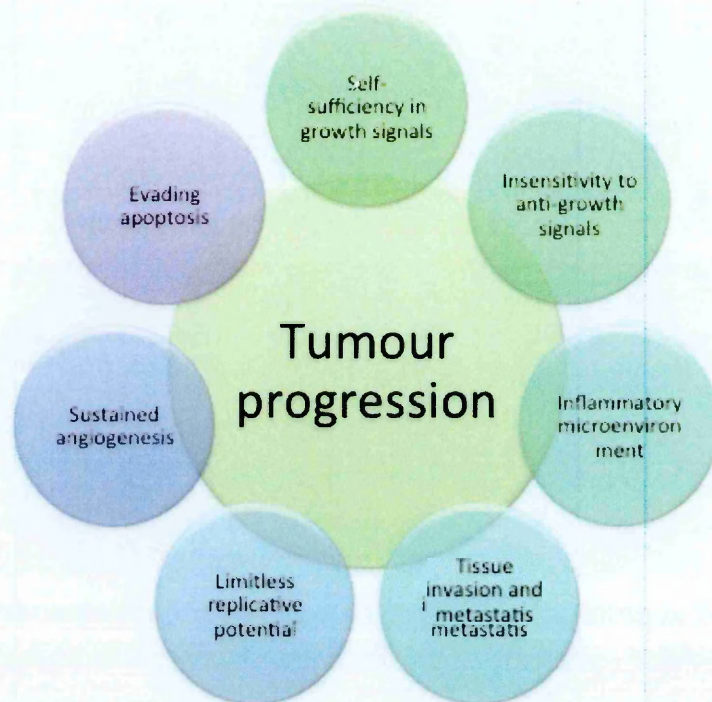


Figure 1. The seven hallmarks of cancer. This picture represents the peculiar and complementary capabilities that allow tumour growth and dissemination. *Adapted from Colotta, 2009 [39]*

Chronic inflammation is likely to be one of the primary causes of several human cancers including colorectal cancer. CRC represents a paradigm of the link between cancer and inflammation. Epidemiologic and clinical studies indicate that patients affected by IBDs (Ulcerative colitis or Crohn's disease) have an increased risk of developing neoplasia, with an increased incidence rate of 2.75 and 2.64 of CRC in patients with ulcerative colitis and Crohn's disease, respectively [40-43]. The mechanisms involved in this process are still poorly understood.

Recently, immune cells and their mediators have been shown to have a pivotal role in triggering colitis-associated colorectal cancer (CAC) development through the induction of DNA alterations in intestinal epithelial cells. Indeed, accumulation of activated immune cells, such as neutrophils, macrophages and dendritic cells is combined with the release of reactive oxygen and nitrogen reactive species, which have been demonstrated to induce genomic mutations [44]. In addition, DNA methylation and histone modification are likely to be linked to chronic inflammation [39, 45, 46]. All these events lead to an altered expression of oncogene such as p53, APC, KRAS [47]. After the genomic events, dysplastic cells become target of cell-derived growth factors and cytokines, which promote tumour growth. From the other side, the immune system has been demonstrated to act as negative regulator of tumour growth, controlling dysplastic cells. This happens till a change in the equilibrium selects dysplastic cell clones capable of growing, thanks to growth factors and pro-inflammatory cytokines; at this point, the immune system becomes a cancer promoter [40].

Although IBDs are chronic inflammatory and immune-mediated diseases, CAC and other types of CRC not directly related to inflammation share some similarities; for instance, the progressive sequence of tumour development from the aberrant crypt foci to carcinomas is similar in both. Even genetic and some of the main signalling pathways, such as Wnt, K-Ras, p53, MMR proteins, are common. Obviously, CAC has its specific characteristics, among which there are some pathogenic mechanisms, comprising, first of all, chronic inflammation and injury-dysplasia carcinoma; the latest does not develop from a prior formed adenoma. However, the presence of an inflammatory infiltrate and molecules appears even in CRC not directly related to IBDs [48].

Immune response to tumours

Tumour context is a multifaceted and dynamic environment, where stromal cells, fibroblasts, endothelial cells, innate cells and lymphocytes interact each other and with tumour cells. The balance of these close interactions results in tumour growth and spread. Therefore, the cross-talk between tumour cells and their microenvironment is extensively considered critical for cancer progression and prognosis, with a special reference to immune infiltrating cells [49].

It has been well established that a leukocyte infiltrate is present in most tumours. It has been generally accepted that the immune system can protect the host against tumour progression through mechanisms of immunosurveillance [50]. Some of the main mechanisms whereby the immune system limits cancer include: the prevention of an inflammatory environment that provide a suitable milieu for tumour development by the elimination of pathogens and resolution of inflammation; recognizing the so called “tumour antigens”, proteins expressed on the tumour cell surface that make them different from their normal counterpart, thus eliminating tumour cells [51]. However, depending on the type of infiltrating immune cells, the effect on tumour progression is different and, according to the cancer type, they can inhibit or enhance tumour growth [52]. The “seven

hallmarks” of cancer have been recently modified by Hanahan and Weinberg, including “avoiding immune destruction” as emerging hallmark, in addition to CRI [38, 52].

The immune infiltrate in human colorectal cancer

The heterogeneity of tumour immune infiltrate is due to the diversity of cell types and where these cells localize in the tumour tissue, both parameters changing according to the tumour type. All immune cell types may be present in a tumour; they can be localized in the core of the tumour, at the invasive margin or in tertiary lymphoid tissue (TLT). Macrophages, mast cells, granulocytes and myeloid-derived suppressor cells (MDSCs) are usually present within or surrounding tumour beds, both in the core and at the invasive front of the tumour. Tumour-infiltrating lymphocytes are localized in specific areas. NK cells have often a stromal localization that does not favour a direct interaction between NK and malignant cells. B cells can be observed both at the invasive margin of tumours and in tertiary lymphoid tissue (TLT) [53, 54]. T lymphocytes can be found at the invasive front and also within the tumour core and even in TLT.

In the colon, an immune infiltrate is already present in benign adenomas and it significantly increases in CRC tissues. Immune cells are localized both at the periphery and in the tumour stroma, occasionally invading cancer cell nests. Most represented leukocytes are T lymphocytes and macrophages, although some B cells, neutrophils, eosinophils, mast cells, NK cells and rare DCs can be found [55-59].

The prognostic value of the TNM staging system has been recently challenged [60]. One of the evidence that lead to review the reliability of the TNM has been that patients with poorly invading but node-positive CRC have a better prognosis than patients with deeper invading but node-negative CRC. As a

complementary and integrative prognostic method, the quantitative assessment of the immune response has been proposed [61], as I will discuss later.

Tumour-infiltrating lymphocytes in CRC

Overview on lymphocyte development and activation

The immune system consists of a wide range of distinct cell types: lymphocytes have a central role because they confer antigen-specificity and are therefore responsible for adaptive immune responses. In my thesis, I first focused on T lymphocytes, which have important regulatory and effector roles, and then on B lymphocytes, specialised cells that produce antibodies. [62]

Lymphocytes develop from stem cells in the bone marrow. All lymphocytes go through complex maturation stages during which they express antigen receptors and acquire functional and phenotypic features of mature cells. B lymphocytes mature in the bone marrow, while T lymphocytes migrate to and mature in the thymus. After cell maturation, they leave the bone marrow or the thymus, enter the blood stream, and reach secondary lymphoid organs. These mature cells are called naïve lymphocytes. Naïve B and T lymphocytes are mature cells that have not been stimulated by antigen to become differentiated lymphocytes. Upon antigen encounters, lymphocytes differentiate into activated cells that have functions in protective immune responses. Activated lymphocytes include CD4+ T helper cells, CD8 + cytotoxic T lymphocytes (CTL) and antibody-secreting B cells. Differentiated helper T cells provide help to B cells to proliferate and produce specific antibody and stimulate inflammation. Differentiated CTLs develop granules containing proteins that kill virus-infected and tumour cells. B lymphocytes differentiate into cells that actively synthesize and secrete antibodies. Some of the progeny of antigen-stimulated B and T lymphocytes differentiate into memory cells, whose function is to mediate rapid and enhanced responses to second or subsequent exposures to antigens. Memory cells may survive in a functionally quiescent or slowly cycling state form many years after the antigen is eliminated. [62]

T cell population is usually identified by the marker CD3 that is expressed on the cell surface at all stages of T cell development. Most of B cells are characterised by the expression of CD20 marker.

Function and their prognostic relevance in CRC

As in melanoma [63] and in ovarian cancers [64], tumour-infiltrating lymphocytes (TILs) limit tumour growth and are associated with improved prognosis [65, 66] also in CRC [61]. Several studies confirmed that the presence of TILs, in particular intraepithelially, is beneficial for patient outcome, being associated with earlier tumour stage, decreased local recurrence rate after surgery and improved overall and disease-free survival, both in metastatic and non-metastatic patients [66-68].

Galon et al. analysed TILs by gene expression profiling and immunohistochemistry; they looked at the type, density and localization of cells in a large cohort of CRC patients [61]. They demonstrated that the adaptive immune response favours patient survival and prevents tumour recurrence in both stage II and III patients. Moreover, they found a dominant cluster of genes involved in Th1 immune responses that inversely correlated with tumour recurrence. Pages et al. investigated the role of TILs in early metastatic invasion, a process characterized by the presence of VELIPI (vascular emboli, lymphatic invasion and perineural invasion). They found that VELIPI-negative patients present a huge immune infiltrate, including effector memory T cells, and a strong Th1 response, both evidence resulting in a prolonged survival [69].

Laghi and colleagues focused their attention on CD3+ TILs and they evaluated them at the invasive front of the tumour in stage II and III CRC patients [60]. According to the above studies, CD3+ TILs are associated with a lower risk of metachronous metastasis, thus predicting prolonged survival. Notably, this is true only in stage II patients (node-negative) and has no longer statistical significance in stage III patients (node-positive), i.e. tumours that have already spread to distant organs, raising the hypothesis that the immunosurveillance capability of CD3+ TILs is compromised in advanced stage tumours [60].

Camus et al. with the expression “immune coordination” wanted to resume the concept of coordination of the intra-tumour immune response. They have demonstrated that the higher is the grade of coordination the better is the prognosis. A higher coordination is associated with a high percentage of tumour-infiltrating T cells. In patients with a low density of these cells, there is impairment in the immune coordination, thus a poorer prognosis [70]. Moreover, they have found a positive correlation between a high density of tumour-infiltrating T cells and late memory and cytotoxic phenotypes of CD8+ T cells and even with a strong Th1 response. All these elements are crucial for the elimination of migrating malignant cells. As long as the equilibrium of the immune coordination at the primary tumour site is solid, metastasis can not occur. To sum up, tumour-infiltrating T cells have been clearly demonstrated to have a protective role in CRC.

Tumour-infiltrating B lymphocytes in CRC

While the role of T lymphocytes in cancer and their relationship with prognosis have been deeply assessed, the role of B cells in the tumour microenvironment and their impact on clinical outcome are still debated. Besides antibody-mediated effects, antibody-independent mechanisms such as antigen presentation, cytokine production, direct cytotoxicity and indirect effects through modulation of other immune cell types have been proposed to be of importance [71-74].

Studying autoimmune diseases through murine models has helped in identifying B regulatory cell (Breg) subsets with immune suppressive functions (**Figure 2**), including B cell subsets producing IL-10 and TGF- β , which can mediate the recruitment and expansion of T regulatory cells [72, 75]. Scientists are making many efforts in order to investigate whether similar immune suppressive B cell subsets migrate to the tumour and acquire an immune suppressive phenotype, potentially leading to the attenuation of the local anti-tumour immune response [76].

Experimental evidence has shown that B cells have a dual role in cancer: on the one hand they can produce antibody or act as antigen-presenting cells (APCs), promoting the anti-tumour activity of tumour-infiltrating T cells [77-80]; on the other hand, the production of antibodies and cytokines can foster tumour development in a paracrine fashion [81, 82]. In particular, B cells play a key role in the maintenance of an immunosuppressive environment, modulating the polarization of the myeloid compartment in a pro-tumour direction. They exert this function by producing cytokines, such as IL-10 [72, 83] and PDL-1 [73], and antibodies that can form immunocomplexes, in turn activating the Fc gamma receptor on myeloid cells [84].

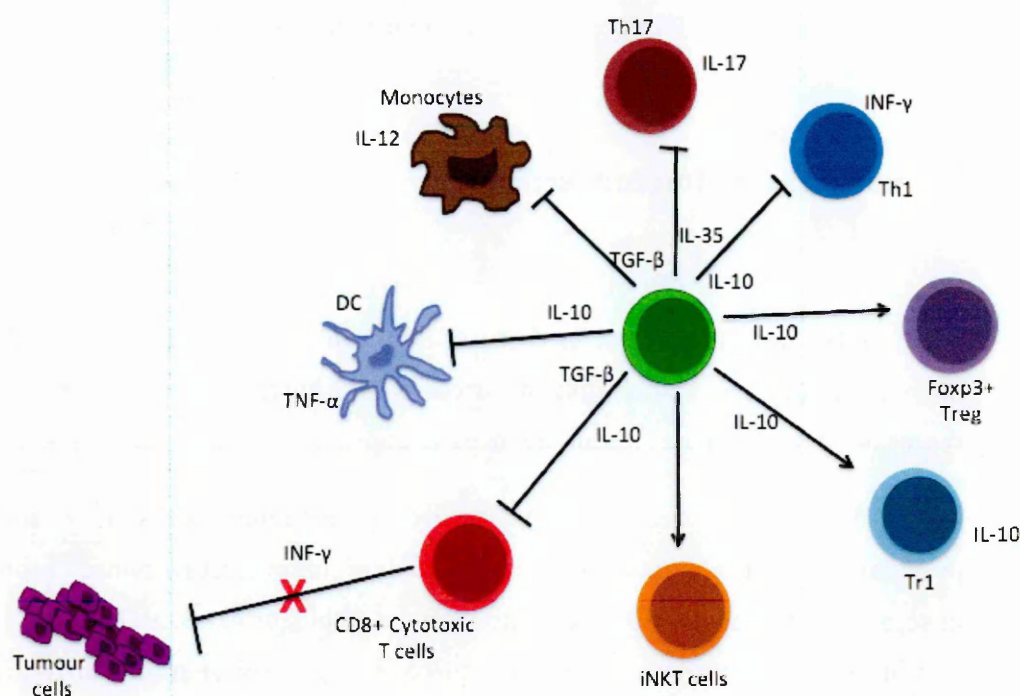


Figure 2. Functional properties of B reg cells. Schematic representation of the main functional features of B reg cells. They produce suppressive cytokines, such as IL-10, TGF-β and IL-35, through which they suppress the differentiation of pro-inflammatory leukocytes, including IL-12-producing DCs, TNF-α producing monocytes, Th17 cells, Th1 cells, cytotoxic CD8+T cells. Moreover, B reg cells can stimulate the differentiation of immunosuppressive T cells, Foxp3 +T cells and Tr1,

and contribute in the maintenance of iNKT cells. *Adapted from Rosser., Immunity 2015 [85]*

The literature aiming to define the role of B cells in colorectal cancer is still limited. A recent work from our group analysing the occurrence of B cells infiltrating human pancreatic adenocarcinoma has shown a peculiar duality of B cell spatial distribution in the microenvironment [74]. Specifically, the prognostic function associated to B cells infiltrating pancreatic cancer markedly changes according to the distribution of B cells in aggregates rather than randomly scattered in the microenvironment, thus suggesting that their spatial distribution in the tissue highly impacts on their function. There are few recent studies showing a characterization of the B cell infiltrate in colorectal cancer [71, 86], but a solid common idea on B cell role in this tumour is still lacking.

Biomarkers and Immunoscore

In the first chapter, I have discussed the canonical classification of CRC and how the prediction of the clinical outcome is achieved. Usually, after surgical resection of the primary tumour, histo-pathological evaluation is performed.

The current staging system, based only on tumour invasion parameters, provides limited prognostic information and does not predict response to therapy. In some patients, advanced-stage cancer can be stable for years, and although rare, partial or full regression of metastatic tumours can occur spontaneously [50]. In contrast, relapse, rapid tumour progression and patient death is associated with 25% of TNM I/II stage CRC patients, despite complete surgical resection and no evidence of residual tumour burden or distant metastasis [50]. The TNM staging focuses primarily on tumour cells and views tumour progression as a cell-autonomous process without considering the host immune response [87, 88].

As I mentioned above, the tumour microenvironment influences cancer progression in different ways, depending on the tumour type and individual characteristics, thus being an important factor that cannot be ignored. Furthermore, the importance of the host immune system in controlling tumour progression has profusely emerged. Nonetheless, the current system does not include it as an element to evaluate during tumour classification.

Histo-pathologic analysis of tumours have shown that the immune infiltrate is not randomly distributed: TILs are found within dense infiltrates in the centre of the tumour (CT), at the invasive margin (IM) of tumour nests and, as it has been recently demonstrated, in adjacent TLT. Therefore, inflammatory and lymphocytic cells, together with other elements of the tumour microenvironment, contribute in establishing the biology of the tumour. The analysis of the location, density and functional orientation of different immune cell populations, named “the immune contexture” [52] (**Figure 3**) in large cohorts of human tumours has allowed the identification of the components that are beneficial for patients and those that are deleterious [87, 89]. Since tumour features and immune reactions are connected, the emerging idea is to assess these factors and to investigate the effects of tumour-host interactions on clinical outcome and prognosis. In human CRC, the presence of TILs is associated to a favourable prognosis [61]. Pages et al [69] demonstrated that the presence of a high density of tumour infiltrating effector memory T cells correlates with the absence of early signs of metastasis. Moreover, time to recurrence and OS strongly correlate with the in situ adaptive immune reaction [61, 69, 90, 91].

Evaluation of the histo-pathological immune reaction may provide novel information on prognosis and identify patient cohorts more likely to benefit from immunotherapy [92, 93].

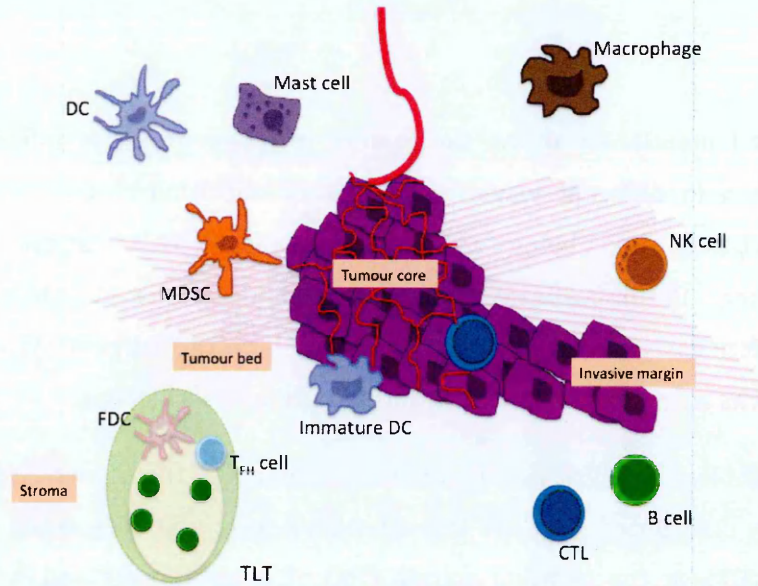


Figure 3. The “immune contexture”: schematic view of immune cell populations and immune structures (TLT), which should be included in the analysis of tumour features and could represent biomarkers of patient outcome. *Adapted from Fridman, Nat Rev Imm 2012 [52]*

Given the weakness of the TNM system and the important value of the immune cells, new systemic and local immunological biomarkers can be a significant innovative strategy in the prediction of outcome and response to therapy. Data demonstrating the impact of immune-classification in several human cancers are constantly growing; immune classification has a prognostic value that may strengthen the value of the TNM classification providing more information and facilitating clinical decision. The clinical translation of the studies demonstrating the prognostic value of the immune contexture in primary tumours is the establishment of an Immunoscore, a grading of tumours based on immune-histologic features.

Galon et al. have proposed it as a useful tool based on the combined evaluation of memory and cytotoxicity markers for the prediction of tumour recurrence and survival in early-stage (TNM I and II) patients with CRC [94]. Specifically, the Immunoscore is based on the evaluation of two lymphocyte populations (CD3/CD45RO, CD3/CD8 OR CD8/CD45RO), both in the CT and in the IM of tumours, as a clinically useful prognostic marker in colorectal cancer. The Immunoscore provides a score ranging from immunoscore zero (I0), when low densities of both cell types are present in both regions, to Immunoscore four (I4), when high densities are found in both regions. This test has a dual advantage: first, it appears to be the strongest prognostic factor for DFS, DSS and OS, including at early-stage CRC; second, it has a biological meaning (adaptive immune response to tumours) and provides a tool or a target for novel therapeutic approaches, including immunotherapy (as recently shown in clinical trials boosting T cell responses with anti-CTLA4, anti PD1, anti PDL-1 [34, 94]). The Immunoscore approach has been applied to 2 independent cohorts (n=602). Only 4.8% of patients with a high I4 relapsed after 5 years and 86.2% were alive. In comparison, 72% of patients with a low score (I0 or I1) experienced tumour recurrence and only 27.5% were alive at five years. These I0 and I1 patients potentially could have benefited from adjuvant therapy, had the Immunoscore been incorporated into the tumour staging [94].

BASIC ASPECTS OF COLON ANATOMY AND MUCOSAL IMMUNOLOGY

Basic aspects of colon anatomy

A considerable part of my thesis has been focused on the identification and characterization of lymphoid tissue in the human and murine colon mucosa. This analysis would not have been possible without a detailed knowledge of the colon anatomy and of the mucosal immune system. For this reason, I am going to provide a summary of the information I learnt, as a background to the later experimental part.

The human colon is part of the large intestine, together with the cecum, appendix, rectum and anal canal. It shares its basic structure with the remaining gastrointestinal canal. It is composed of four principal layers from the lumen outward:

- the mucous layer (mucosa)
- the sub mucosa
- the muscle layer
- the serous layer

Each layer has a predominant tissue type and a specific digestive function.

The mucous layer is covered by a simple lining epithelium with some important functions: to provide a selectively permeable barrier between the contents of the tract and other tissues and to facilitate the transport and digestion of food. The cells in this layer produce mucus and numerous digestive enzymes. Below the epithelium, there's a *lamina propria* of loose connective tissue rich in blood and lymph vessels and smooth muscle cells, sometimes containing also glands, many lymphoid cells and lymphoid aggregates (part of mucosal associated lymphoid tissue, MALT) important in defence against bacteria. The third component of the mucosa is the *muscularis mucosae*, a continuous thin layer of smooth muscle separating the mucosa from the sub mucosa. It promotes the movement of the mucous layer independently of other movements of the digestive tract, increasing its contact with the food.

The sub mucosa is composed of loose connective tissue containing elastic fibres, many blood and lymph vessels and the sub mucosal nerve *plexus* (or Meissner's *plexus*). It may also contain glands and lymphoid tissue. Lymphoid aggregates are frequently found crossing the muscularis mucosae and invade the sub mucosa.

The external muscle layer contains smooth muscle cells, spirally oriented, divided into two sub-layers according to the main direction the muscle cells follow, the myenteric nerve plexus (Auerbach's plexus) and blood and lymph vessels. The serosal layer is represented by the protective visceral peritoneum. It is composed of loose connective tissue, rich in blood and lymph vessels and adipose tissue and presents a simple squamous covering epithelium (mesothelium).

Mucosal immunology

The large number of commensal bacteria and potential pathogens, shielded by only a single layer of epithelial cells within the intestinal lumen, is a continuous extraordinary challenge for the intestinal immune system [95]. In the human gut, trillions of bacteria are present, including more than 400 species, that feed themselves with alimentary products that an individual ingests, thus grow and proliferate. However, this "infection" is restrained. Indeed, bacteria are useful for the human organism, which allows them to grow taking advantage of their metabolic products as nutrients, as promoters of the intestinal absorption and as protective agents against pathogens and cancer. According to the complexity of the human gut flora and to the extension of the gut mucosa, that opens a wide gate for facing different pathogen agents [96], the intestinal immune system is huge and complicated, concomitantly challenged to fight against pathogens and to restrain the intestinal flora in the gut lumen.

An equilibrium between the gut flora and the intestinal immune system is needed to ensure the digestive and absorptive processes [97, 98]. Luminal bacteria neither cross the epithelial barrier nor seriously impair this equilibrium; at the same time, the intestinal immune system does not damage the “good” bacterial population. However, changes in dietary habits, ingestion of toxic compounds or infection with pathogens can perturbate this harmony, leading to alterations of the composition of the gut flora, damage to the epithelium, infection of the intestinal tissues and/or induction of inflammation.

The intestinal immune system is equipped with several “soldiers”, specialised in the maintenance of the equilibrium, including both single cell types and organised lymphoid aggregates, also known as gut-associated lymphoid tissue (GALT). Among the main cellular populations, there are members of the innate and the adaptive immune system, such as intra-epithelial lymphocytes (IELs), dendritic cells (DCs), macrophages, mast cells, plasma cells and lamina propria leukocytes (LPLs). Besides immune cells, there are specialised epithelial and mesenchymal cells. All these elements constantly interact with each other to target bacteria in the gut lumen, in the epithelium or in the lamina propria. In particular, dendritic cells (DCs) sample antigens or live bacteria from these compartments and migrate to the T-cell zones of inductive sites, such as mesenteric lymph nodes and the Peyer’s Patches (PP). There, DCs can induce adaptive immune responses that lead to the generation of effector T cells and of plasma cells that produce IgA, the main immunoglobulin that is manufactured by the immune system and secreted in large amounts into the gut lumen [95].

GALT is defined as an anatomically discernible lymphoid structure and can be viewed as an array of small lymphoid organs lining the intestinal tract. It is composed of highly specialised lymphoid organs, Peyer’s Patches (PPs), and small lymphoid aggregations, Criptopatches (CPs) and isolated lymphoid follicles (ILFs). These organs constitute an adaptive response unit that monitors immunologically relevant events and maintains either tolerance or activates adaptive immune reactions [99].

CPs have been hypothesized to play the role of primary lymphoid organs, supporting the extrathymic development of T lymphocytes, as well as to be precursor of mature ILFs, while PPs and MLNs are well defined secondary lymphoid organs involved in the induction of the mucosal immune response. In contrast, ILFs are tertiary lymphoid structures whose function is still under debate [100].

CPs and PPs develop during the embryonic life, starting from the recruitment of lymphoid tissue inducer (LTi) cells to specific sites of the developing intestine. This, in turn, stimulates the recruitment of dendritic cells, T cells and B cells, to organize the immune response to the microbiota. IELs colonise the epithelium before birth. Post-natally, after the colonisation of the neonatal intestine by the microflora, PPs complete their functional maturation and CPs develop into mature ILFs [101-103].

ILFs are present both in human [104] and mice [103]. Several clusters (100-200) can be found throughout the length of the anti-mesenteric wall of the mouse small intestine, as well as in the colon. ILFs are morphologically and functionally similar to the follicular unit that composes a PP and represent an alternative and inducible site for the induction of intestinal IgA antibody responses [103, 105]. As mentioned above, ILFs development is triggered by environmental stimuli after birth, in particular by commensal bacteria or inflammatory stimuli. Their precursors, CPs, are phenotypically different and are mainly constituted by lineage negative cells (lin-), namely cells that do not express CD3, CD19, CD11b, CD11c but express SCF-R, cKIT and IL-7R. Several studies have demonstrated plasticity of these aggregates. In germ-free mice, the majority of lymphoid aggregates are very small, devoid of B cells, thus defined as CPs; after the colonisation by the microflora from SPF (Specific Pathogen Free) mice, the composition of lymphoid aggregates changes, with the maturation from CPs to ILFs. Nonetheless, the number of these structures does not change and it is fixed during the first week of life [105].

The observation that mice lacking PPs could still produce and secrete IgA in response to luminal antigens led many to believe IgA CSR (Class Switching Recombination) could occur outside of organized lymphoid tissues [106, 107]. However following the identification of ILFs, these observations could be reinterpreted to support a role for ILFs in IgA production [108].

In inflammatory conditions, the number, diameter and density of ILFs increase, showing their plasticity and adaptability to the continuously changing intestinal environment. They participate not only to the immune surveillance, but also to the normal mucosal regeneration of the colon and they are likely to play a role in colorectal carcinogenesis [53, 109], as it will be discussed later.

SECONDARY AND TERTIARY LYMPHOID TISSUE

Lymphoid organs

Over the past century, the importance of the existence of highly organised primary and secondary lymphoid tissues has been deeply highlighted. Since the immune system is extremely dynamic and its functioning is based on continuous interactions between diverse cell populations, the presence of specialised immune sites - lymphoid organs -allowing these peculiar interactions for the development of an effective immune response, emerges as very important.

The developmental process of lymphoid organs depends on the expression of specific molecules in a specific time frame during ontogenesis [110]. They can be classified in primary, secondary and tertiary lymphoid organs. The thymus and the bone marrow (liver in the fetal period) constitute the primary lymphoid organs, where the largest part of lymphocyte development occurs. In mammals, T lymphocytes both develop and mature in the thymus, while B cells develop in the fetal liver and in the adult bone marrow. During T and B cell differentiation, lymphocytes acquire their repertoire of antigen-specific receptors that represent the key point for adaptive immune response to pathogens in the periphery.

As to secondary and tertiary lymphoid organs, they share functional and architectural features, which need to be understood in order to perceive the relevance of investigating the occurrence of tertiary lymphoid tissue in tumours, which ultimately represented the aim of my thesis. For this reason, I will dedicate a section to the development and function of secondary and tertiary lymphoid organs and their similarities.

Secondary lymphoid organs (SLOs)

Secondary lymphoid organs include the spleen, lymph nodes (LNs) and the mucosa-associated lymphoid tissue (MALT), which includes PPs and ILFs. All these structures are essential for the induction of immune responses against pathogens,

providing an ideal milieu for cellular and molecular interactions that lead to activation, differentiation and maintenance of lymphocytes, and facilitating the interaction between the immune system and microorganisms [111].

Three processes are strongly favoured by the presence of SLOs: first, they restrain pathogen spreading throughout the body, by organizing an adaptive immune response. Second, APCs sample antigens in the periphery and bring them into SLOs. Here, APCs meet their cognate pathogen-specific lymphocytes, giving rise to an efficient antimicrobial immune response. Third, SLOs are a reservoir of all the factors needed for survival and differentiation of lymphocytes [111].

Lymphoid organs develop with a complicated process during embryogenesis. A general model for lymphoid organogenesis is shared among SLOs, even if each organ needs different signals to completely form.

"it is the pervading law of all things... that the life is recognizable in its expression, that form ever follows function". Based on this moral, Louis Sullivan designed the modern skyscraper, characterised by a strong steel frame. He wanted these buildings to provide an efficient microenvironment where people can easily travel through and connect with others, in every part of the structure. Likewise, SLOs are supported by a network of stromal cells that contribute to the division of the organ in specialised areas and to the connection of these. Thus, SLOs microanatomy accounts for its function, which is to mediate the immune system dynamics aiming at taking on the continuous infectious challenges from the environment [111].

Since the microanatomy of SLOs is similar under homeostatic conditions, I will briefly describe the lymph node (LN) to glance at the microarchitecture of these organs. A fibrous capsule and an underlying sub-capsular sinus surround the cellular contents of the LN. The cortex is made up of B cell areas organised in primary and, after antigen stimulation, secondary lymphoid follicles with GC (Germinal Centre). Inside the cortex there is the para-cortex, which is composed of T cells and DCs. The medulla consists of medullary cords, which are separated by medullary sinuses. The LN vasculature includes HEVs and lymphatic vessels. Lymphocytes circulate constantly through the LN by entering HEVs and exiting via

efferent lymphatic vessels. DCs enter via afferent lymphatic vessels. Lymph with DCs and soluble antigen enters the LN at several points through afferent lymphatic vessels and deposits antigens in the subcapsular sinus.

Vascular and lymphatic endothelial cells are abundant non-hematopoietic cells in SLOs; they show a primary functional structure and they produce molecules, such as cytokines, through which they contribute in the dynamics within the LN. Specialised vascular endothelial cells of HEVs have a peculiar morphology and express molecules, such as PAd (CD62L) and CCL21, that are extremely important for lymphocyte entry in the LN. Lymphatic endothelial cells express Lyve1, several adhesion molecules and CCL21, all involved in lymphocyte entry in lymphatics.

Once in the LN, cell location is “orchestrated” by lymphoid chemokines. CXCL13, expressed in the B cell follicles, guides B cells to them; CCL19 and CCL21, expressed in the T cell zone, positions T cells and DCs in the para-cortex. In addition, lymphoid chemokines expressed on HEVs facilitate the recruitment of lymphocytes to LN [112].

Therefore, secondary lymphoid organs represent a complex scenario in which immune responses take place; this scenario can be different depending on the pathological context. Therefore, SLO structure defines their function, but also the function influences the structure. The actors of the immune response can set up their stage: they adjust SLO microarchitecture depending on their needs during the life of the host [111].

Secondary lymphoid tissues develop during embryogenesis, thus during a specific and pre-determined moment, as well as in specific and predetermined places. Therefore, the time frame and the anatomical localization where they develop are always the same (**Figure 4**). Moreover, their formation does not depend on any antigenic nor inflammatory stimulus, but it represents a physiological process.

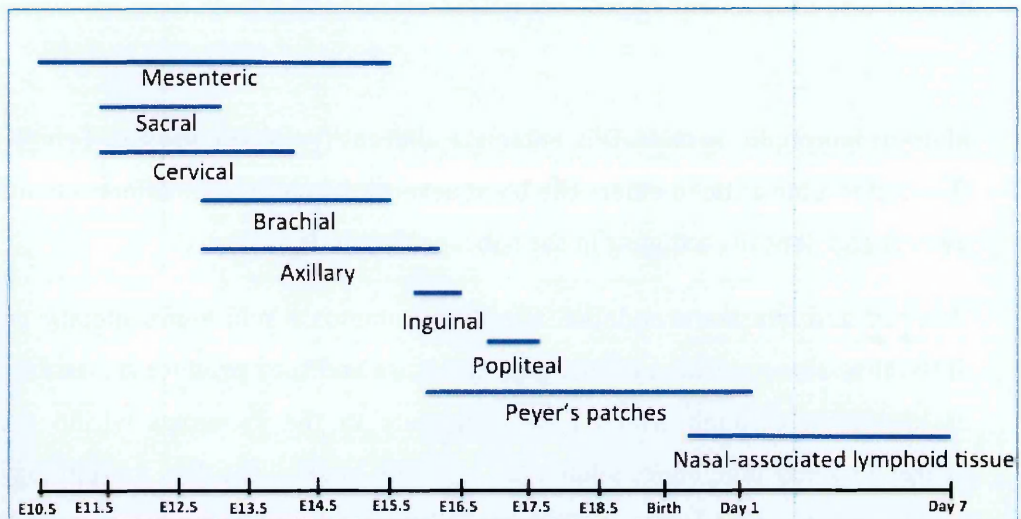


Figure 4. Time-line of lymphoid organ development during ontogenesis. This picture shows that lymphoid organs do not all develop at the same time, but sequentially. *Adapted from Mebius, 2003 [110]*

Tertiary lymphoid tissue (TLT)

In contrast to SLOs, tertiary lymphoid tissues develop during the adulthood as a consequence of chronic inflammatory conditions, chronic infections or autoimmunity. As mentioned above, their formation is named lymphoid neogenesis. TLT can potentially develop in every part of the body: it does not appear in pre-determined nor predictable sites. Contrary to LN, TLT does not have an external capsule: this is an important feature, favouring antigen uptake in the peripheral tissues. Molecular and cellular mechanisms that control lymphorganogenesis during ontogeny have been deeply studied and identified, whereas, little is known about cells and pathways involved in lymphoid neogenesis. The frequent occurrence of TLT in inflamed tissues suggests a role for inflammatory mediators. Inflammation is a localised and thinly regulated response to an injury, tissue destruction and/or infections. Acute inflammation is an innate immune response, usually rapid and self-limiting. However, sometimes it does not resolve, turning into a persistent/non-resolving condition, which is chronic

inflammation. This state promotes several pathological conditions, such as autoimmunity and cancer.

A focal characteristic of local inflammation is the interaction between immune cells and stromal cells that usually leads to resolution of the inflammatory response. During chronic inflammation, inflammatory mediators secreted by stromal cells lead to immune cell recruitment, activation, survival and, in this case, retention at pathological sites, with the persistence of the inflammatory lesion [113]. Buckley et al. assume that chronic inflammation involves immune cells being positioned in the wrong place at the wrong time.

During an inflammatory response, immune infiltration is usually seen as diffuse immune cells spread throughout the inflamed tissue. However, it is now well established that immune cells can also form organized aggregates in the targeted inflamed tissue, and these aggregates can take part to the developmental process of the pathologic state [112, 114-116]. The organized aggregates developing in peripheral non-lymphoid organs in pathologic conditions are called "ectopic lymphoid-like structures" (ELs) or tertiary lymphoid tissue (TLT). They share the structural organization with secondary lymphoid organs, showing compartmentalized B cell-rich and T cell-rich areas, specialized DC populations, activated stromal cells and HEVs. The phenomenon of TLT formation is termed lymphoid neogenesis [115, 117] (**Figure 5**).

Pathologists have established that some criteria have to be fulfilled to define the presence of TLT: the presence of segregated areas of B and T cells; FRCs (Follicular Reticular Cells) within the T cell zone; PNA⁺ or MECA79⁺ HEVs; evidence of B cell class switching or GC reaction in B cell area; expression of the enzyme AID; FDC (Follicular Dendritic Cells) within B cell zone. [117].

Studies on human pathologies and murine models have shown that there are at least three main events leading to lymphoid neogenesis during chronic inflammation: the expression of inflammatory cytokines, such as tumour-necrosis factor (TNF) and lymphotoxin (LT), the production of lymphoid chemokines by stromal cells and the presence of HEVs [112].

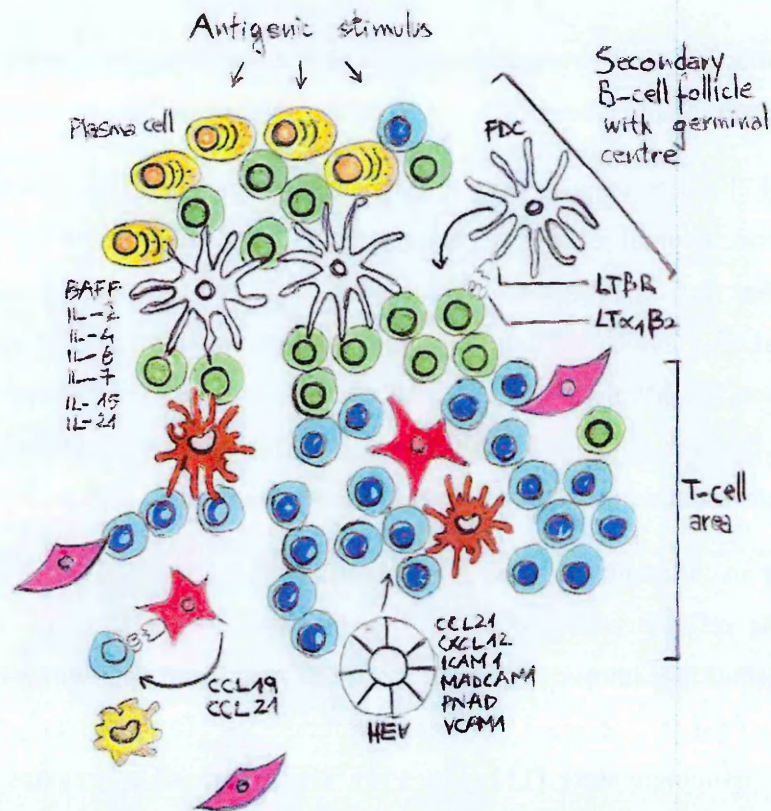


Figure 5. Schematic model of cellular and molecular networks involved in lymphoid neogenesis. This picture represents the leukocyte populations and the key molecules involved in lymphoid organogenesis. The schematic lymphoid organ shows a T cell area (blue light cells) and a B cell follicle, including FDCs and plasmacells. FDCs have a pivotal role in the recruitment of B cells, producing CXCL13, and in their activation. FDC: follicular dendritic cells. *Adapted from Aloisi, 2006 [115]*

Since TLT phenotypically appears similar to a secondary lymphoid organ, it has been wondered if the same processes occurring in TLOs, such as B and T cell priming or clonal expansion, can occur also in TLT. Does ectopic lymphoid tissue have a function? Can it contribute to the exacerbation of chronic inflammatory

disease or in the protection of the host against pathogens? These questions have sharpened studies focused on lymphoid neogenesis in several diseases. [114, 115].

Cellular and molecular determinants in lymphoid organogenesis.

As mentioned above, a general scheme that summarizes the developmental program of lymphoid organs has been elaborated. One of the crucial and earliest events in lymphoid organ formation is the interaction between two cell types: a haematopoietic lymphoid-tissue inducer cell (LTi) and a non-haematopoietic stromal lymphoid-tissue organizer cell (LTo), that respectively express the surface markers CD45+CD3-CD4+ IL-7R+c-kit+ and VCAM+ICAM+ [110]. The encounter takes place in the anlage (or primordium) that is an embryogenic active niche, where these cells find a favourable microenvironment in which they accomplish their function.

LTi cells derive from common lymphoid progenitors in the fetal liver; the absence of the CD3 marker distinguishes them from lymphocytes. LTi cells express the chemokine receptors CCR7 and CXCR5 that promote their homing to those places where lymphoid organogenesis takes place.

LTo cells express lymphotoxin- β receptor (LT β R) that binds its ligand lymphotoxin- $\alpha_1\beta_2$ (LT $\alpha_1\beta_2$) expressed on LTi cells. This binding leads to the release of lymphoid chemokines, including CCL19, CCL21, CXCL13, and the expression of adhesion molecules, fostering the recruitment of others LTi and lymphocytes. As B and T cells also express LT $\alpha_1\beta_2$, a positive feedback loop is created that leads to the local accumulation of more B cells and T cells [116, 118]. From one hand, LT $\alpha_1\beta_2$ expression controls the lymphoid chemokine production by stromal cells, from the other hand, lymphoid chemokine signalling on lymphocytes guarantees the expression of LT $\alpha_1\beta_2$ on these cells. Blocking this loop in anywhere, leads to a defective lymphoid organ development and architecture [116] (Figure 6).

It has been demonstrated that the injection of a soluble LT β R-immunoglobulin fusion protein during embryogenesis can block the development of lymphoid organs; and, depending on the timing of the injection, all or some SLOs do not develop, showing that lymphoid organs do not all develop at the same time, but sequentially [119].

LT is a multifunctional cytokine included in the TNF family. It has a similar structure to that of TNF α ; indeed both molecules contribute to lymphoid organogenesis and the regulation of inflammatory and immune-regulatory responses. LT also controls the differentiation of stromal cells and HEVs and is important for the expression of several adhesion molecules on vascular endothelial cells and stromal cells. For example, LT α , LT $\alpha\beta$ and TNF signalling through their receptors control the expression of MAdCAM, PNAd, VCAM-1 and iCAM-1. Therefore, LT signalling has a key role in many aspects of SLOs development, lymphoid architecture and cell dynamics. The importance of its role has been confirmed studying the knock out murine model for LT α . This mouse lacks LN, PP and has an impaired architecture in the spleen [120].

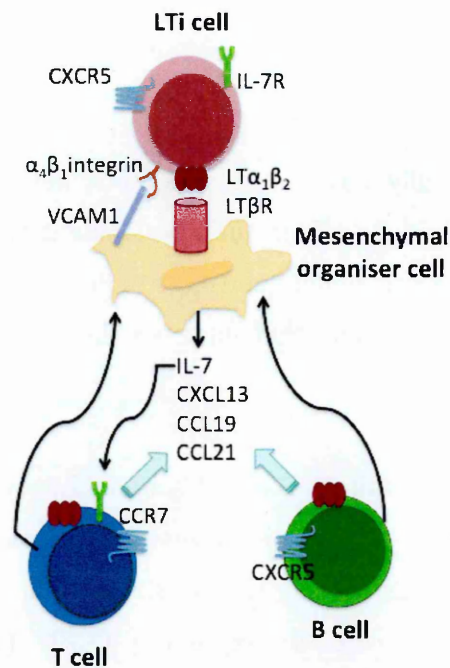


Figure 6. Schematic representation of the pivotal molecular and cellular events involved in lymphoid organogenesis. Lymphoid organogenesis is initiated by the interaction of lymphoid tissue inducer cells (LTi) with mesenchymal organiser cells. The first step includes the recruitment of LTi cells to mesenchymal cells through CXCR5 and the connection through VCAM1 and VLA4. As a consequence, LT $\alpha_1\beta_2$, expressed by LTi, interacts with LT β R on LTo. This interaction induces local expression of lymphoid chemokines (CCL19, CCL21, CXCL13), which, in turn, attract T and B cells to the activation site. Since B and T cells also express LT $\alpha_1\beta_2$, they contribute to generate a positive feedback loop that leads to the local accumulation of more B and T cells and to the development of the organised microenvironment of SLOs. *Adapted from Junt, 2008 [111]*

Although the majority of the developmental program of TLT is similar to that of SLOs, the mechanisms that trigger TLT formation are poorly defined, and for this reason studies focus on specific elements in each disease, from lymphoid chemokines, to cytokines as LT, to specific cell populations.

Presuming that SLOs and TLT organogenesis share the majority of molecular mechanisms, genetically modified mice for molecules involved in this process have been useful to study their role in the development of both SLOs and TLT. These models have allowed to understand that molecular mediators involved in TLT formation are mostly the same that guide lymphoid organogenesis.

An interesting aspect of the process of lymphoid neogenesis is the occurrence of lymphoangiogenesis [112]. Lymphatic vessels are associated to immune infiltrate, including B and T cells, raising the possibility that lymphatic endothelial cells actively recruit lymphocytes by expressing CCL21 [121]. Therefore, lymphatic neoangiogenesis contributes to the recruitment of infiltrate and even in the maintenance of a potentially detrimental alloreactive immune response in renal transplants, suggesting that lymphoangiogenesis and lymphoid neogenesis are connected. This suggests that lymphatic vessels could be a new therapeutic target, for instance, in autoimmune diseases, where TLT is likely to sustain the pathology [112]. Additionally, another field of therapeutic approach would be in cancer, where an increase of lymphatic drainage promotes metastasis [122].

TLT and pathology

Based on the pathological setting in which TLT occurs, the initial trigger can be of different nature, In general, it is recognized that tertiary lymphoid tissue develops during chronic inflammatory processes associated to autoimmune diseases [123, 124], chronic infections [125] and cancer [53, 126, 127](**Figure 7**). Once established TLT presence and its morphological features, the next question would

be whether it has a pathophysiological role in diseases. The function that TLT acquires depends on the organ in which it occurs and on the pathological context. The local microenvironment controls the development and function of TLT [127]. From one hand, it has been shown to support disease progression, acting as an alternative site for autoreactive lymphocyte recruitment and activation [128]; from the other hand, it can “protect” the organism by creating an immune response that restrains the pathological process [129]. Overall, detailed studies have shown that TLT shares with SLOs not only the microarchitecture but also functional properties, directing several B cell and T cell responses, including the induction of effector functions, clonal expansion, antibody generation, somatic hypermutation, affinity maturation, immunoglobulin class-switching [114, 115]. I will analyse different pathological settings in which TLT has been observed and which function it acquires in each disease.

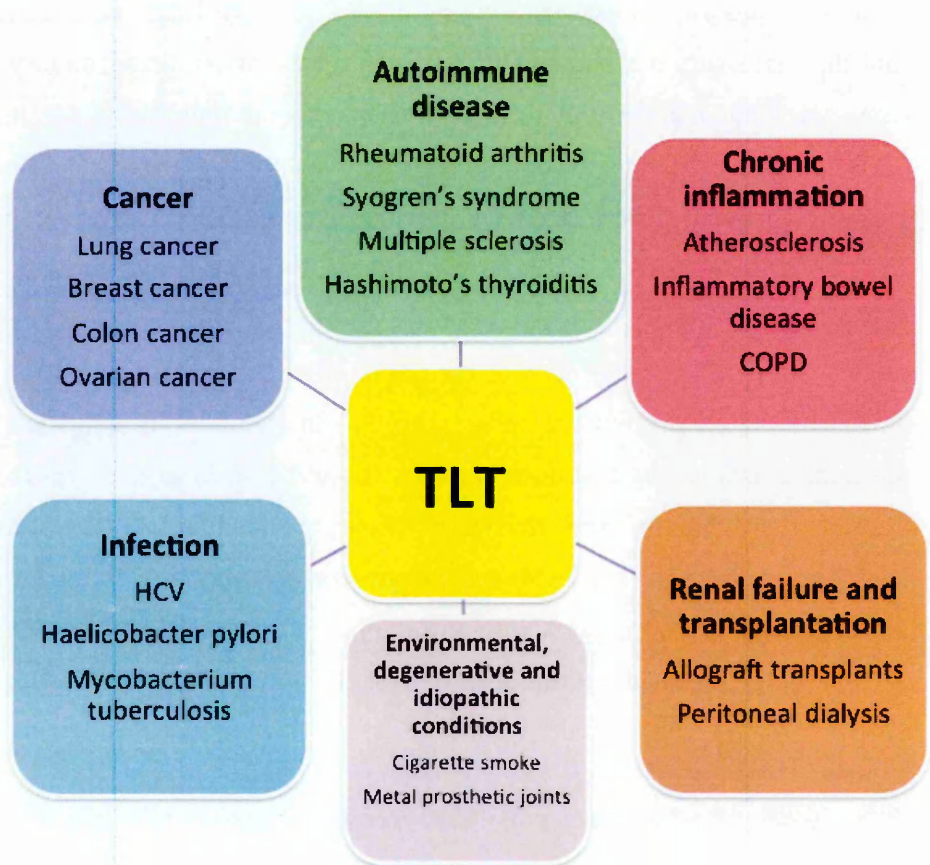


Figure 7. TLT in human diseases: the schematic representation shows groups of diseases in which the occurrence of TLT has been documented.

TLT in Autoimmune diseases

The presence of lymphocyte aggregates with the appearance of functionally ectopic immune sites has long been described in the inflamed target organs or tissues of patients who are affected by usually organ-specific autoimmune diseases, including the synovial tissue in rheumatoid arthritis [124], the meninges in multiple sclerosis [130], the salivary glands in Sjogren's syndrome [131], the thymus in Myasthenia gravis [132] and the thyroid gland in Hashimoto's thyroiditis [133]. In these clinical disorders, TLT develops in response to disease-specific auto-antigens, which also guarantee the maintenance of this lymphoid structure within the inflamed tissue [114]. Therefore, in the majority of autoimmune diseases, TLT presence correlates with the in situ production of autoantibodies and an exacerbation of disease severity. It has been demonstrated that the regulatory mechanisms that govern tolerance within secondary lymphoid tissues are not maintained in TLT associated to autoimmunity. For instance, in SLOs, autoreactive B cells that recognize self-antigens may be excluded from entering germinal centres and they downregulate CXCR5, thus not responding to CXCL13 [134]. Instead, TLT allows autoreactive B cells to entry in the target organ [135]. Here, B cells can differentiate into high-affinity autoreactive plasmacells that release autoantibodies directly in the organ affected by the disease, for examples anti-citrullinated protein antibodies (ACPAs) in rheumatoid arthritis [136], and antibodies against the ribonucleoprotein Ro and L in Sjogren's syndrome [137], against thyroglobulin and thyroperoxidase in Hashimoto's thyroiditis [133]. However, despite all the evidence demonstrating that TLT is an immunologic active site present in patients with autoimmune diseases, it is still hard to define if TLT development leads to the disease or if the autoimmune disease leads to TLT [116].

TLT in Chronic infection

Several viral or bacterial infections are associated to chronic inflammation that provides a strategy to avoid the spread of the infection to the whole organism. Lymphoid neogenesis in chronic infections is likely to be evolved as a new mechanism whereby containing the infective reaction where it arises. Therefore, infection-associated mucosal TLT can mount protective immune responses in situ [114, 115]. TLT occurrence has been documented in chronic hepatitis C virus (HCV) infection [138, 139], in H. Pylori-induced gastritis [125], in Lyme disease [140].

The infection of mice with H.pylori (HP) triggers the formation of lymphoid aggregates with GC in the gastric mucosa [129], while influenza virus and M.Tuberculosis induce lymphoid neogenesis in the lungs of rodents [141]. These data suggest that TLT, at sites of infection, drives a protective immune response in addition to, and often independently from, SLOs. However, a failure to eradicate a pathogen has considerable clinical implications and can lead to the development of autoimmunity (ex, chronic HCV infection) or lymphoma (ex HP associated chronic gastritis) [114].

TLT in Cancer

As I have discussed above, it is now well known that immune cells infiltrate human solid tumours, although the prognostic value of immune cells in cancer patients is variable according to the tumour type and stage of disease [52]. Overall, targeting immune infiltrating cells by immunotherapeutic approaches is one of the main goal of cancer research today [142].

In cancer, the presence of TLT has been documented in many tumours [143, 144], including lung [127] [145], melanoma [146], ovarian [147], colon [53, 92, 148], breast [149, 150] and germ cell cancer [151]. However, not every patient with a specific type of cancer develops TLT and when it is present, its contribution to disease varies considerably. TLT develops more frequently in some tumour types

than in others, suggesting that certain tumours provide a microenvironment that appears as a permissive milieu for lymphoid neogenesis. Moreover, TLT can show different levels of organization in different tumour types, changing from patient to patient. Such differences may reflect different stages in the program of lymphoid neogenesis, as previously suggested in non-transformed inflammatory settings. Finally, the density of TLT is heterogeneous even among tumours of a given type, once again reflecting the diversity of individual tumour microenvironments, being more or less permissive to lymphoid neogenesis [152].

The emerging evidence that the adaptive immune responses can be triggered independent of secondary lymphoid organs has been encouraging scientists to extend their knowledge of TLT. Depending on the tumour type and stage, TLT could have different roles. From one hand, the presence of TLT in tumours may represent an ectopic immune site where tumour antigens can be presented and the anti-tumour immune response can take place. From the other hand, the pro-tumour role of chronic inflammation suggests that TLT may also represent a source of growth factors and pro-angiogenic factors that sustains tumour progression. Moreover, lymphatic vessels associated to TLT may provide an additional way for cancer cell to spread to other parts of the organism.

One of the first reports of the occurrence of TLT in human cancer came from a study on breast cancer. Coronella et al. focused her attention on infiltrating ductal carcinoma (IDC) of the breast and investigated whether tumour-infiltrating B cells (TIL-B) represent a tumour-specific immune response [126]. They highlighted the heterogeneity of the lymphocytic infiltration with particular attention to the B cell component, which is present only in the 24% of the adenocarcinomas, but when present, it represents up to 40% of the TIL population. Interestingly, B cells appear as dense aggregates [153] that are often localized in stromal areas immediately adjacent to tumour nests. Lymphoid aggregates resemble lymph node germinal centres. The majority of B cells are IgG+, in contrast to the IgA+ population normally present in healthy breast tissue. Moreover, the B cell zone is surrounded by T cells and within B cell follicle there are CD21+ FDCs. Each characteristic makes these lymphoid aggregates good candidate as alternative immune site

where B cells can get activated. Indeed, Coronella et al. demonstrated that TIL-B undergo tumour antigen-driven expansion in intra-tumour aggregates.

In 2008 Dieu-Nosjean reported the presence of TLT in non-small-cell lung cancer (NSCLC) [127]. The highly compartmentalization of TLT within lung tissue suggested that it could mediate an on-going immune response. As a potential mechanism for TLT formation, Authors showed that the more tumour were infiltrated by mature DCs expressing DC-LAMP, the more T and B cells were organized and proliferated in TI-BALT (Tumour Induced-Bronchus-Associated Lymphoid Tissue), suggesting a pivotal role for DC cells in TLT development. Moreover, a DC-Lamp high tumours had a higher density of TILs and accordingly high density of DC-Lamp+ DCs was associated with a favourable clinical outcome for patients with early-stage NSCLC.

Therefore, TLT seems to represent an active immune site, able to recruit T lymphocytes at the tumour site and to activate immune cells in the tumour microenvironment. Moreover, its density, when accurately assessed, can represent a prognostic biomarker. Thus, TLT assessment and targeting emerge as promising approaches for the design of novel prognostic immune signatures and immunotherapeutic strategies.

MATERIALS AND METHODS

Patients. We considered tissue specimens from 351 stage II and III patients without any sign of metastatic disease at diagnosis, who consecutively underwent radical surgical resection for pT3 or pT4 colorectal cancer (CRC). Patients' demographics, clinical and histopathological data were available and obtained from the Institutional Intranet. The equipe of clinicians who provided us the specimens confirmed that the absence of metastases at diagnosis had been assessed in all patients by combining histopathological findings, surgical records and perioperative imaging, according to institutional protocols. Similarly, to study the prediction of disease recurrences according to the state of immune infiltration, patients with pT1 or pT2 CRC, who have a very low risk of progression at diagnosis, and patients with perioperatively detected metastases were excluded. To exclude potential confounders in the study design, stage I patients were not enrolled in the cohort studied for their unlikely occurrence of disease recurrence, while stage IV CRC patients were not included because they are characterized by the presence of distant metastases, which is an outcome event of our analysis. Patients who underwent neoadjuvant radiotherapy for rectal cancer were excluded from the study, because of the possibility of interference with the assessment of the local immune response. Chemotherapy treatment was administered and allocated by a non-random assignment, according to adjuvant protocols in use at the time of surgery.

Study design. We retrospectively studied tissue specimens of CRC patients who consecutively underwent radical surgical resection for pT3 or pT4 colorectal cancer (CRC) at the Humanitas Clinical and Research Center, Rozzano, Milan, Italy, from January 1997 to November 2005. Investigators who were blinded to the results of the morphological analysis assembled a clinical retrospective database by collecting demographics, clinical and histopathological data from the institutional intranet. These variables, together with the median values of CD3-TLT, CD3-TIL, CD20-TLT and CD20-TIL IRA%, were tested as predictors of patient's outcome. The outcome of patients who undergo radical resection of colorectal cancer is a variable affected by an event defined as any local tumour

recurrences or any metachronous distant-organ metastasis and named disease free survival (DFS). To detect or exclude any postsurgical tumour recurrences, patients underwent thoraco-abdominal computed-tomography (CT) abdominal ultrasonography, and chest radiography, that were done according to common protocols for surveillance. The observation period started immediately after the surgical procedure. Time to follow-up was stopped at the time of the death of patient for any case unrelated to colorectal cancer disease, and this case was not considered an event of outcome. The mean follow-up period of the cohort studied was 4.71 years (SD = 2.63 years) for DFS. The detection of tumour recurrence or death was computed from diagnosis until data were censored on May 30, 2010. To further assess any possible biases, interaction analyses in predicting patient's prognosis were performed for all the variables assessed in order to detect any effect modifier ($P < 0.10$).

Immunohistochemistry. From each patient enrolled in the study, 2 μm thick tissue slides from formalin processed and paraffin embedded tumour sections were processed for immunohistochemistry. After deparaffinization and rehydration, sections were immersed in an antigen retrieval bath, incubated with 3% H_2O_2 for 15 minutes. Slides were autostained (IntelliPATH FLX, Biocare Medical) with primary antibodies raised against CD3 (clone F7.2.38, Dako), CD20 (clone L26, Dako), DC-Lamp (clone 1010E1.01, Dako), PNA⁺ (MECA-79, BD Pharmingen), Lyve-1 (ab14917, Abcam), CD21 (clone EP3093, Abcam), α -SMA (clone 1A4, R&D) CXCL13 and CCL21 (AF801 and AF457, R&D), IgA and IgG (polyclonal rabbit; Dako), Ki67 (MIB-1, Dako), BCL6 (PG-B6p, Dako), IL-10 (polyclonal goat; R&D), PDL1 (rabbit polyclonal; abcam). A 30 minutes incubation with the DAKO Envision system (Dako) or Anti-Goat Polymer kit (Biocare) followed. Diaminobenzidine tetrahydrochloride (DAB) (Dako) was used as chromogen. Nuclei were lightly counterstained with a freshly made haematoxylin solution (Meditex). Presence of fibrosis was assessed on 2 μm -thick sections stained for 20 min with 0.1% Sirius red in saturated picric acid (Sigma-Aldrich). The sections were further washed in water, mounted and analysed under an optical

microscopy. Slides were digitized using a computer-aided image analysis system (Olympus DotSlide, Olympus, Italy). TLT exhibited a distinct structural organization in CRC, outlined by an area composed of CD3⁺ cells and a compartment of CD3 negative lymphoid cells (CD20+ B cells). To quantify immune cells, I randomly selected three non-contiguous microscopic areas located at the tumour invasive front occupied by either CD3-TLT or CD20-TLT. With the same approach three non-contiguous microscopic areas located at the tumour invasive margin including scattered CD20-TILs were selected at the interface between tumour stroma. Accordingly, cancer tissue in CD20-TILs microscopic areas had to represent approximately 50% of the entire microscopic field. Computer-assisted measurement of the selected areas was obtained as the percentage ratio between immunoreactivity and the total digitized tissue surface. For each histological section, the mean values obtained in three different regions were calculated and used for the subsequent statistical analysis. Median values of the overall distribution of the immune variables were chosen as representative cut-off to perform statistical analyses.

Statistical analysis. The association between the extent of the immune variables, patient's baseline characteristics and tumour features was estimated by Pearson's simple linear regression analysis. A Cox proportional hazards model was developed to assess the role of the density of immune variables and other demographic, clinical and histopathological features, in predicting the occurrence of disease specific survival (DFS). To assess for confounders, COX multivariate analysis was performed by entering only variables and their significant interactions with a P value less than 0.20 at univariate analysis. Interactions between variables were calculated by analysing their multiplicative term in the Cox model. By a backward stepwise elimination approach, non-significant variables, and their non-significant interactions, were removed from the model. Interacting variables at multivariate analysis (P<0.10) were then tested for subgroups analysis accordingly. Differences in median values of immune variable density between subsets of CRC and DFS were tested by the Mann-Whitney U test

and by Cuzick's trend test. Kaplan-Meier curves of DFS were plotted, while log-rank test was used to compare the curves of each subgroup of CRC patients. For each test, only two-sided P values lower than 0.05 were considered statistically significant. All the analyses were done using Epi Info (Version 3.4.3), StatsDirect Statistical software (Version 2.5) and GraphPad Prism software (Version 4.1).

Quantification of HEV in human CRC. Consecutive tumour slides from 20 CRC patients were stained with antibodies raised against PNAd. For each tumour slide, the absolute numbers of PNAd positive vessels within each intratumour follicle and follicles associated to the normal mucosa were quantified.

Mice and murine models of CRC. 8-week-old C57BL/6J mice were purchased from Charles River (Calco, Italy); μ MT mice and eGFP/C57BL/6 mice from Jackson Lab. Procedures involving animals and their care were conformed to Institutional Guidelines in compliance with National and International laws and policies. Mice were housed in a specific pathogen-free animal facility of the IRCCS Humanitas Clinical and research Center in individually ventilated cages. In the AOM/DSS model, mice developed adenomas as a result of the combined treatment with the carcinogen azoxymethane (AOM, 10 mg/kg; Sigma- Aldrich) and sequential administration of the mucosal irritant dextran sulphate (DSS) [3 rounds of 2 % DSS (MW = 36.000–50.000; MP Biomedicals)] in the drinking water. Adenomas develop in the distal colon overtime during the different treatments and are evaluated after 10 weeks. In the genetic APCMin/AOM model, the formation of adenomas is induced by the weekly administration of the carcinogen AOM (10 mg/kg) in APCMin mice, once a week for 4 weeks, followed by sacrifice at 9 weeks. In both models, mice develop adenomas in the distal colon overtime during the different treatments and are evaluated after 10 weeks. In the implanted model, CMT93 murine CRC cell line is injected subcutaneously. After three weeks, mice develop tumours.

Immunofluorescence. Frozen sections of murine colons or colon whole mounts were stained with specific antibodies: B220 (clone RA3-6B2, e-bioscience) CD3 (clone 145-2C11, e-bioscience), CD11c (clone N418, e-bioscience), CD21 (ep3093, Abcam), FDC (FDC-M1, BD Pharmingen), CXCL13 (polyclonal goat IgG; R&D), Lyve1 (rabbit polyclonal, Abcam), PNA^d (MECA-79, BD Pharmingen), MadCAM (MECA-367, BD Pharmingen), CD31 (clone 2H8, Millipore), CCL21 (clone 59106, R&D systems), CD8 (clone 4SM15; eBIOSCIENCE)

The three-dimensional visualization of B cell follicles was obtained on colon whole mounts; briefly, colons were removed from mice and immersed in 1% paraformaldehyde fixative solution overnight. Tissues were then washed and stained immunohistochemically by incubating whole mounts with primary antibodies diluted in PBS containing 0.3% Triton X-100, 2% bovine serum albumin, 5% normal goat serum, 0.01% glycine and 0.1% sodium azide overnight, followed by overnight incubation with secondary antibodies. The three-dimensional visualization of TLT in mMT mice was obtained on colon whole mounts by 3D visualization of cellular aggregates evidenced by Syto 13 Green Fluorescent Nucleic Acid Staining (Invitrogen).

Morphometric Analysis. Morphometric analysis of colon whole mounts was performed by 3D visualization of B220⁺ follicles. Isosurfaces were obtained by importing confocal RGB image stacks to Imaris software. For each colon analysed, one stack of each follicle identified was acquired and analysed. CD3⁺ cell enumeration was performed by computer-assisted image analysis on 20 µm thick sections of whole colons.

Cell purification and transfer. Single-cell suspension of splenocytes from eGFP/C57BL/6 mice (Jackson Lab) was prepared, after red blood cell lysis. Cells were then washed, resuspended in PBS at a concentration of 10×10^6 and injected into the retro-orbital sinus of recipient animals. Mice were sacrificed after 24 hours; colons were opened and frozen flat. GFP+ cells within TLT were enumerated on 20 μ m thick frozen sections by confocal microscopy.

Enzyme Linked Immunosorbent Assay (ELISA) for murine immunoglobulins.

ELISA was used for the detection of immunoglobulins (Ig) in murine stool supernatants. Stool were collected, weighed and suspended in PBS and 0,05% sodium azide. Samples were submitted to sonication; then supernatants were recovered. Ig were measured with specific antibodies against various isotypes (anti-IgA, anti-IgM, anti-IgG1, anti-IgG2b, anti-IgG3) provided by SouthernBiotech. The protocol was performed as follows:

1. Coating with coating antibody (goat anti-mouse Ig) in carbonate buffer at 4°C overnight;
2. Discard the coating antibody solution and wash the plate with PBS-Tween;
3. Block the plate with blocking solution (PBS/BSA 1%) for 1 hour at room temperature;
4. Discard the blocking solution and wash the plate;
5. Add mouse stool supernatant in duplicates to the plate and make serial dilutions (1:3) of standard murine Ig (starting at 625 ng/ml). Incubate for 2 hours at room temperature;
6. Discard supernatants and wash the plate;
7. Add HRP conjugated goat anti-mouse isotype specific antibodies to the plate and incubate for 1 hour in PBS at room temperature;
8. Discard and wash;
9. Add Peroxidase substrate solution to the plate. Develop the plate at room temperature in dark;
10. Block the reaction with NaOH 3N and read the plate using an ELISA reader with a wavelength of 405nm;

Lamina propria leukocyte (LPL) purification. The entire colon was removed, chopped with scissors into 1 cm small pieces and washed with HBSS/serum 5%. After washes, colon pieces were transferred into a solution containing 2mM EDTA and incubated for 20 minutes on a rocker (twice). Then, samples were vortex 15 s at maximum setting and tubes were shaken. In order to allow pieces to settle, samples were centrifuged and supernatant were discarded. Then, tissue was transferred to an eppendorf tube and minced very well. Tissue was transferred again to a 14ml tube with Collagenase from clostridium hystoliticum (Sigma Aldrich) solution 0.5mg/ml and put on rocker for 30 min at 37°C. After 30 min, tissue was passed through 21G needle until parts are all gone and then poured through a 70 um cell strainer into a 50 ml tube. Once added RPMI, cells were centrifuged and supernatant was discarded. Pellet was resuspended with 5 ml of Percoll 44% and transferred into 15ml canonical tube. 7ml of Percoll 66% was added on the bottom of the tube, that was centrifuged for 20 min at 2000 rpm (brake low). Once recovered cells, they were washed with RPMI and centrifuged for 6-7 min at 1500 rpm (at least twice). Cells were ready for FACS staining or mRNA analysis.

FACS analysis. Flow cytometry was performed using FACS Canto II flow cytometer and the final analysis with FlowJo software (TreeStar). LPLs were washed with FACS Buffer (PBS/BSA) and resuspended in a blocking solution containing mouse Fc Block (anti-mouse CD16/CD32 monoclonal antibody – BD bioscience). After blocking, cells were washed again and stained with several antibodies: anti-mouse CD45/PerCP (clone 30 F11; eBIOSCIENCE), anti-mouse CD19/APC (clone MB19-1; eBIOSCIENCE), anti-mouse B220/PEcy7 (clone RA3-6B2; eBIOSCIENCE), anti-mouse CD3/FITC (clone 145-2C11; eBIOSCIENCE), anti-mouse CD8/PacificBlue (clone 53-6.7; eBIOSCIENCE), anti-mouse NK1.1/APC (clone PK136; eBIOSCIENCE), anti-mouse F4/80/PE (clone A3-1; AbD SEROTEC), anti-mouse Ly6G/FITC (clone RB6-8C5; BD Pharmingen).

mRNA expression analysis. Total RNA was extracted from LPLs using QIAGEN RNeasy Mini Kit.

The reverse transcriptase PCR (RT-PCR) was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacture's instructions. Random primers were used. Applied Biosystems 7900HT Fast Real-Time System was used to detect the expression of several genes. Fast SYBR® Green Master Mix and specific primers (designed by our laboratory) were used to prepare samples.

RESULTS

PART I

T CELL DISTRIBUTION IN COLORECTAL CANCER

Background and goal

This chapter will focus on the characterization of the CD3 compartment in the colonic mucosa of colorectal cancer patients and murine models, both in terms of tissue localization and cellular and molecular composition. As already discussed in the introduction, lymphoid tissue is present in normal colonic mucosa as part of the MALT and mediates important functions, including pathogen recognition, activation of an appropriate protective immune response, which principally involves secretory antibodies, particularly IgA. The aim of the analyses I am going to present was firstly to investigate the occurrence of TLT in the colon mucosa during carcinogenesis and which are the mechanisms driving TLT development. The ultimate goal was to test whether TLT has a clinical relevance in human CRC.

Tertiary lymphoid tissue in human colorectal cancer

Localization of TLT in human CRC

While the presence of lymphoid tissue, also known as isolated lymphoid follicles (ILFs), in the normal colon mucosa has been well documented [103, 105], the occurrence and behaviour of inducible (or tertiary) lymphoid tissue (TLT) during carcinogenesis, has not been deeply investigated.

Since CD3⁺ T cells represent an important component of TLT, to investigate the occurrence of TLT in human CRC specimens, I first evaluated, by immunohistochemistry, CD3⁺ T cell infiltration. In human CRC specimens, CD3⁺ T cells are not only interspersed in the tumour tissue and scattered throughout the stroma as TILs (CD3-TILs) (**Figure 1D**), but they also group into lymphoid aggregates (CD3-TLT), at the bottom of the crypts in normal mucosa (**Figure 1A**), and next to tumour nests (**Figure 1B**) and in stromal region, at the invasive front of the tumour (**Figure 1C**). These aggregates present a high degree of compartmentalization, with B and T cell areas, thus resembling TLT [53].

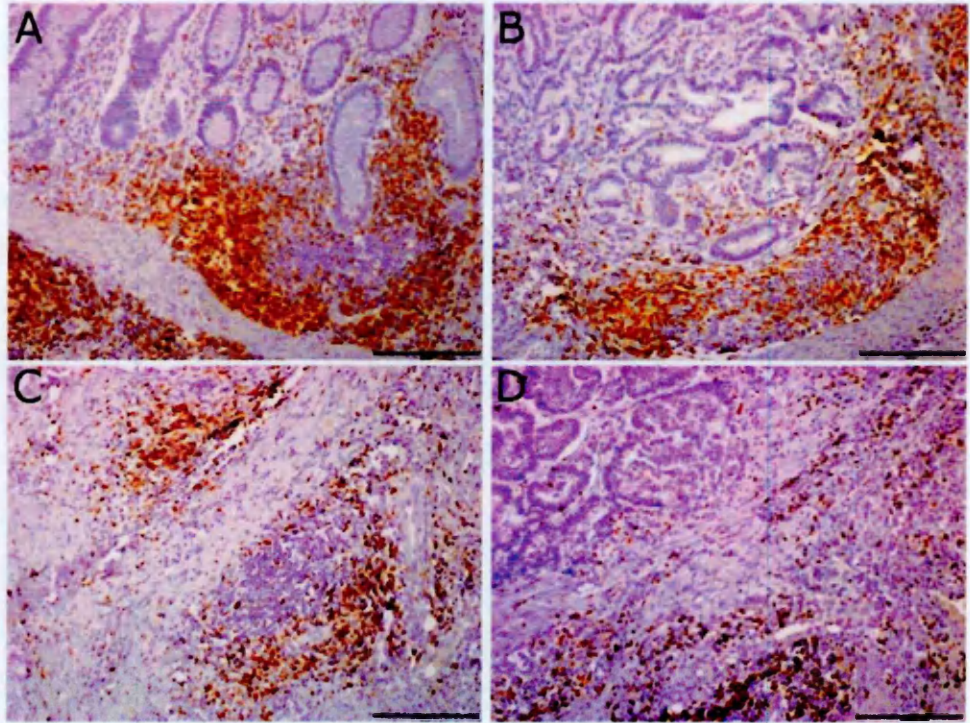


Figure 1. Tertiary lymphoid tissue in human colorectal cancer specimens. Immunohistochemical staining with an anti-CD3 antibody allows identification and distribution of CD3+ T cells in human CRC. CD3+ T cells localize in lymphoid tissue associated to normal mucosa, at the bottom of the crypts (A) and within TLT localized both adjacent to tumour nests (B) and in stromal regions (C); CD3+ T lymphocytes (TILs) are also scattered throughout the tissue as shown in (D). Scale bars: 200 μ m. Haematoxylin counterstaining.

Characterization of TLT in human CRC

Lymphoid tissue associated to normal mucosa has the typical organization of a lymphoid follicle within a secondary lymphoid organ (SLOs). To test whether the architecture of tumour-associated TLT is comparable to that of SLOs, I performed several stainings with specific antibodies that could highlight the presence of important cellular mediators usually found in lymphoid organs. Staining with anti-CD3 (**Figure 2A**) and anti-CD20 (**Figure 2B**) antibodies revealed that tumour-

associated TLT has compartmentalized T (CD3+) and B (CD20+) cell areas; follicular dendritic cells (CD21+) gather together within a B cell zone (**Figure 2C**). Mature dendritic cells are present within TLT, as evidenced by DC-LAMP immunoreactivity (**Figure 2D**), and may contribute to T cell activation [53].

An important feature of lymphoid organs is the presence of a vascular network, including lymphatic vessels and high endothelial venules (HEV), specialized haematic vessels that allow recruitment of naïve lymphocytes [154]. Immunohistochemistry with an antibody against PNAd (**Figure 2E-F**) and Lyve1 (**Figure 2G**), markers for HEVs and lymphatics respectively, was performed to investigate whether TLT is equipped with a vascular network, allowing trafficking of immune cells and the communication with the surrounding tissues.

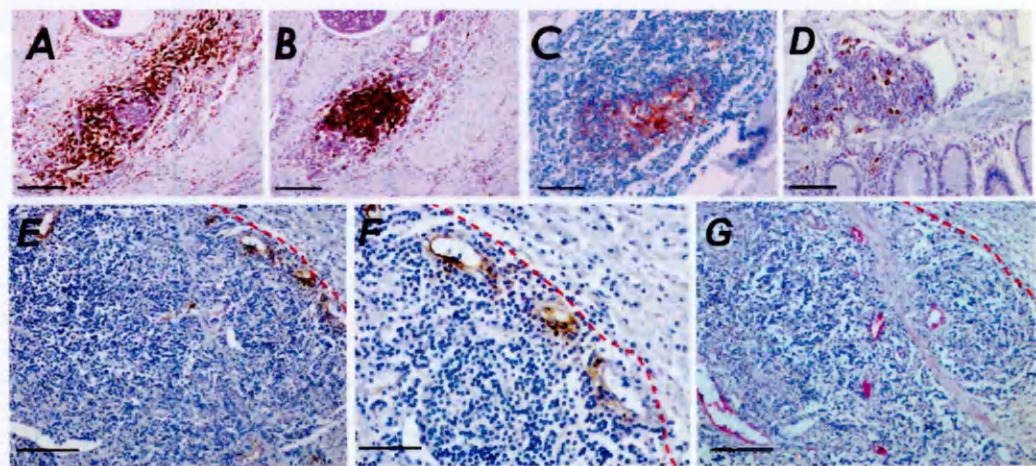


Figure 2. Characterization of TLT in the colonic mucosa of colorectal cancer patients. Immunohistochemistry with antibodies against CD20, CD3, CD21, DC-Lamp revealed that TLT in human CRC specimens is composed of segregated areas of CD3+ T lymphocytes (**A**) and CD20+ B cells (**B**) as shown in consecutive sections; within the B cell zone, a core of FDC CD21+ (**C**) is present. DC-Lamp + mature DCs are interspersed within TLT (**D**). Vessels within lymphoid tissue include PNAd+ HEV (brown in **E-F**) as well as Lyve-1+ lymphatic vessels (pink in **G**), suggesting traffic of naïve lymphocytes. Sections in **E** and **F** are consecutive sections, showing HEV and lymphatic vessels in the same follicle. Dot lines indicate follicle contour. Scale bars: 200 µm (**A, D**), 50 µm (**C-E-F-G**).

Potential mechanisms driving TLT development during colorectal carcinogenesis.

Once established that TLT is a histologically distinct immune component in the microenvironment of human CRC and presents important features of lymphoid sites, including cell subsets and a specialized vascular network, I started to wonder which is the mechanism driving the formation of TLT during colorectal carcinogenesis.

The accurate compartmentalization of TLT suggests the involvement of specific molecular mediators released in the tumour microenvironment. In chronic inflammatory conditions, activated stromal cells are known to produce several inflammatory mediators, including chemokines, responsible for the infiltration of immune cells. Given the association between colorectal cancer and chronic inflammation and since TLT is preferentially localized in stromal regions, to test the hypothesis that stromal factors might be responsible for driving neogenesis of TLT, I analysed by immunohistochemistry the tissue surrounding TLT. I confirmed the presence of activated alpha-SMA+ fibroblasts (**Figure 3A**) surrounding and within TLT, and by Sirius Red, the presence of collagen fibres (**Figure 3B**) in the surrounding tissue. Moreover, I evaluated the presence of lymphoid chemokines CCL21 and CXCL13, which are both expressed within lymphoid tissue (**Figure 3C-D**), indicating active recruitment of cells and plasticity of TLT.

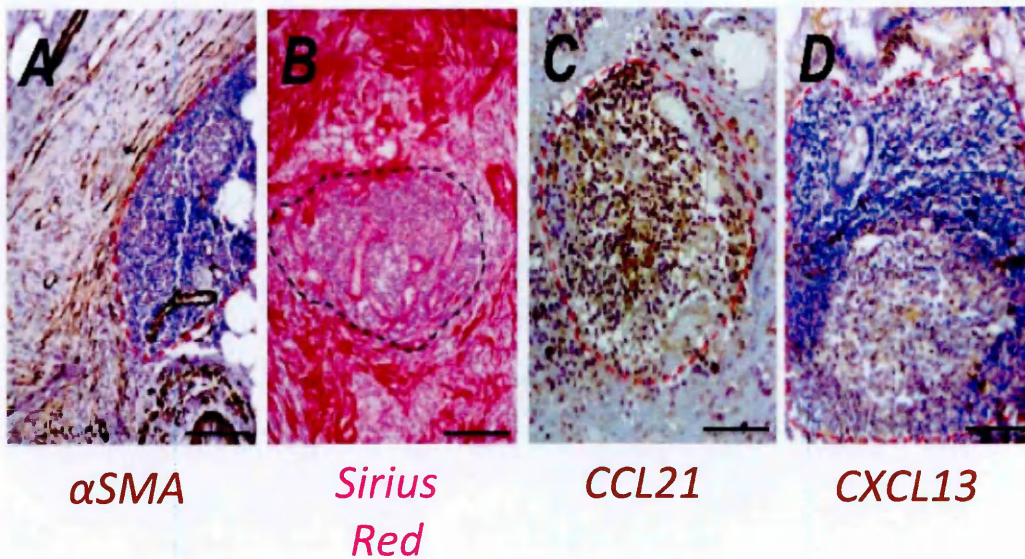


Figure 3. Tumour associated inflamed stroma associates to formation of highly organized intra-tumour lymphoid tissue. Lymphoid tissue in human colorectal cancer specimens is usually located in stromal areas. Many alpha-SMA (a marker of local fibrosis and inflammation) positive cells are present in stromal areas surrounding TLT (**A**); staining with Sirius Red indicates the presence of collagen fibres (**B**). The lymphorganogenic chemokines CCL21 (**C**) and CXCL13 (**D**) are present inside TLT. Dotted-circles indicate intra tumour lymphoid tissue. Scale bars: 200 μ m.

Analysis of High Endothelial Venules in tumour-associated TLT

As I mentioned before, the process of lymphoid neogenesis includes new vessel formation, to allow proper circulation of immune cells. Antigen presenting cells (APCs) reach lymphoid organs through lymphatic afferent vessels, while naïve lymphocytes through high endothelial venules (HEVs), specialized blood vessels, which direct the traffic of naïve T cells [115].

I have shown that tumour-associated lymphoid tissue includes both lymphatics and HEV (**Figure 2** in this chapter). Therefore, to understand whether TLT occurrence was associated to a perturbation of the vascular compartment, I decided to compare the vascular network of lymphoid tissue associated to normal

mucosa and intra-tumour TLT. HEVs were present mostly in the context of TLT and very rarely found in the surrounding tumour tissue. I found an increased number of HEVs in TLT within the tumour compartment ($p < 0.05$) (**Figure 4**).

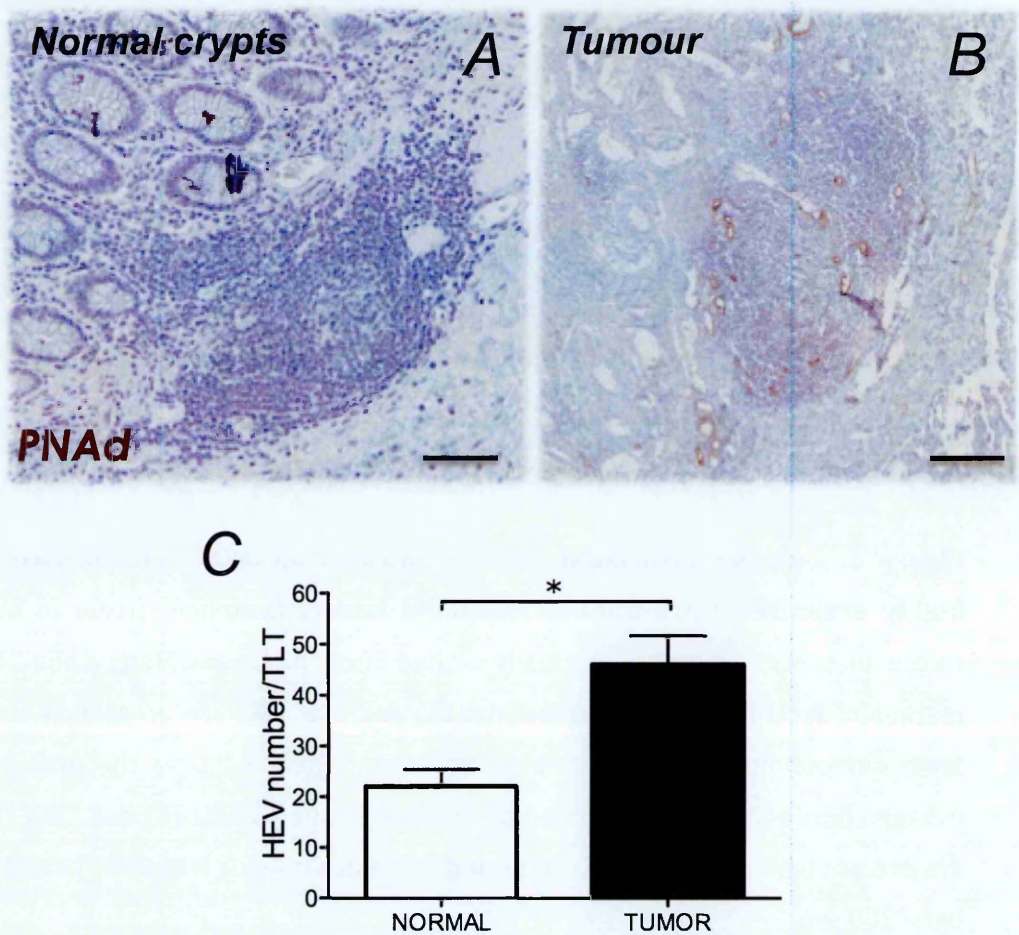


Figure 4. Specialized haematic vessels increase in tumour-associated TLT.

Figure 4 A shows a lymph node-like follicle associated to normal crypts devoid of HEV, while tumour-associated TLT has a high number of PNAd+ HEVs (brown in **B**). PNAd+ HEVs significantly increase in tumour associated TLT compared to those in lymphoid tissue associated to normal mucosa (**C**). HEVs were quantified by computer assisted image quantitative analysis on slides from 20 paraffin embedded cancer tissue specimens stained with an anti-PNAd antibody. Bars in **C** represent the number of HEV in each lymph node like follicle. Scale bars: 200 μ m.

* $p < 0.05$ by Student's T test.

The expansion of HEVs in tumour-associated TLT suggests it may have an active role in lymphocyte recruitment at the tumour site. Furthermore, HEV development is one of the earliest events during lymphoid neogenesis, when lymphocytes have to be recruited to the neo-formed lymphoid organ. In the tumour context, this would mean that intra-tumour TLT develops *de novo*, during carcinogenesis, and it may have active functions in the tumour microenvironment.

Evaluation of the relationship between TLT and tumour-infiltrating CD3+ T cells in colorectal cancer.

While CD3+ T cell density is recognized as a prognostic marker for CRC patients [60, 61], the mechanism for CD3+ T cell recruitment and activation at the tumour site has not been identified.

The expansion of HEVs raises the hypothesis that there could be an association between tumour-infiltrating CD3+ T cells and TLT. It may be that TLT contributes to T cell recruitment at the tumour site in colorectal cancer and the recruitment may be mediated by TLT-HEVs.

To address this point, I systematically evaluated by immunohistochemistry with an anti-CD3 antibody the density of CD3-TLT at the invasive front, in 351 tissue specimens (**Figure 5A**) and quantified the area of CD3-TLT by computer-assisted image analysis. The cohort of patients includes stage II (SII) and stage III (SIII) CRC patients, whose clinico-pathological features are summarized in **Table 1**. Whole-tissue analysis of the CD3+ infiltrate showed a higher density of CD3-TILs in tumours containing high density of CD3-TLT (**Figure 5C**), compared to tumours containing low density of lymphoid tissue (**Figure 5B**). In fact, I found a positive correlation between CD3-TIL density and CD3-TLT density ($R = 0.29$; $p < 0.001$) (**Figure 5D**). Together with the increased amount of HEVs within CD3-TLT, the correlation of CD3-TLT density and CD3-TILs suggests that TLT may be involved in T cell recruitment; this result has important clinical implications, considering the positive prognostic value that CD3-TILs have in human CRC.

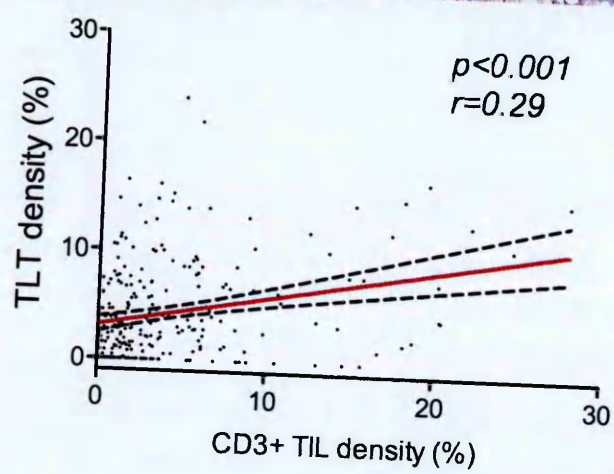
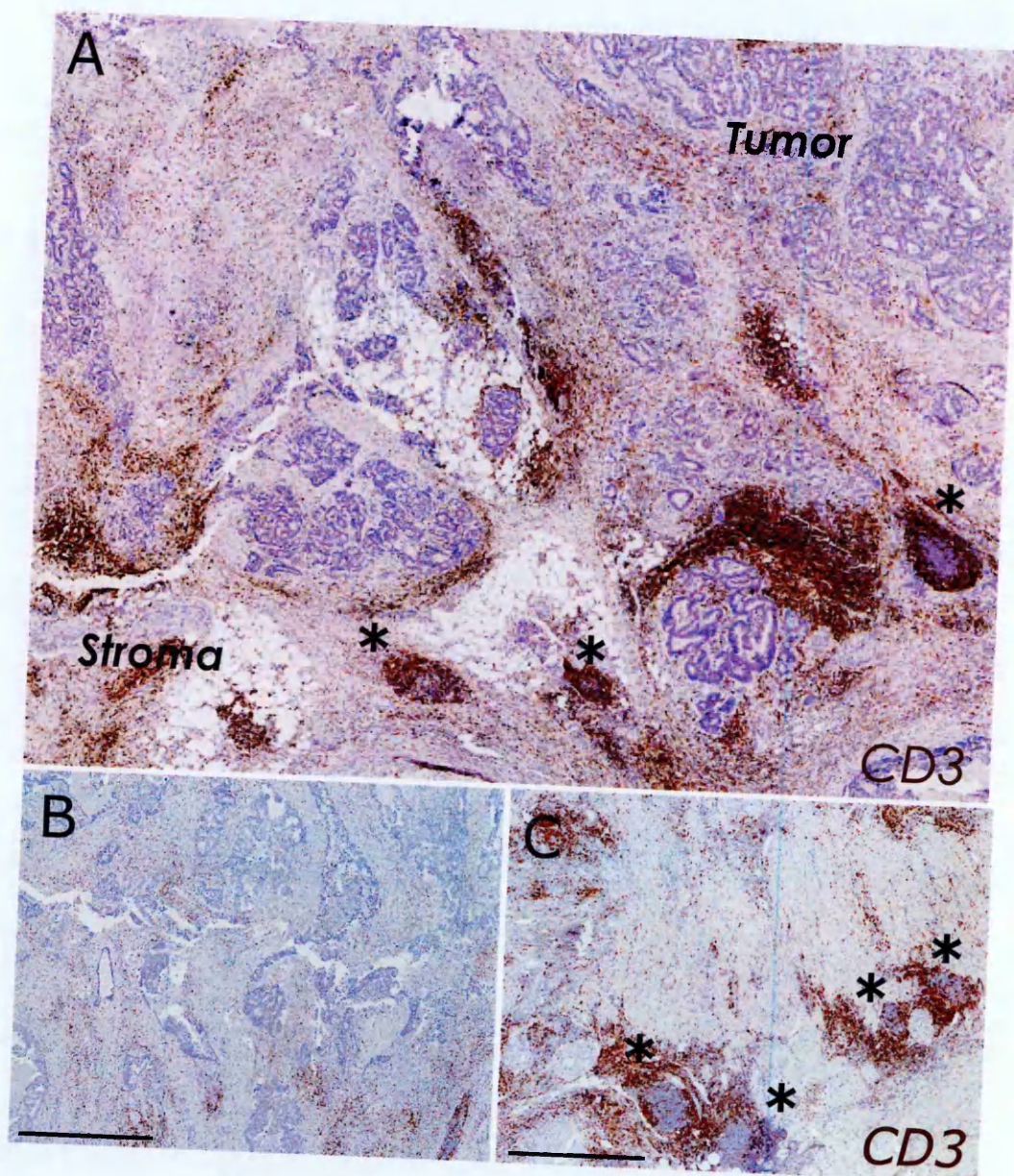


Figure 5. Increased T cell infiltration associated to intra tumour lymphoid tissue. Figure 5 A shows a representative image obtained by virtual scan of a colorectal tumour specimen. Staining with anti-CD3 antibody allows identification of lymphoid tissue. Asterisks indicate TLT in the tumour stroma, at the invasive front. (B-C) representative images of two colorectal tumour specimens with low (B) and high (C) degree of T cell infiltration and TLT. Asterisks indicate TLT. (D) Correlation between CD3+ TILs density and TLT density in 351 CRC patients. Patients with a high density of TLT also have a high density of CD3+ T lymphocytes. p value by simple linear regression analysis. Scale bars: 1 mm

Tertiary lymphoid tissue in murine models of colorectal cancer

Cellular and molecular components of the microenvironment of human CRC allow neogenesis of tertiary lymphoid structures. Tumour-associated TLT develops preferentially in stromal regions, where activated fibroblast producing chemokines and cytokines are present. In our cohort of stage II and III CRC patients, TLT density positively correlated with the density of TILs, suggesting that it may be involved in lymphocyte recruitment.

In order to increase our knowledge on the mechanisms driving TLT development and the role of TLT in T cell infiltration in CRC, I have taken advantage of different preclinical models of CRC. These models have allowed me to elucidate the role of chronic inflammation in triggering TLT formation and to verify the hypothesis that TLT mediates lymphocyte recruitment at tumour site.

Visualization of TLT in the murine colonic mucosa

As in human CRC specimens, I started with localization and characterization of TLT in the murine colon mucosa. Also in the mouse colon, aggregates of lymphocytes are present in homeostatic conditions, as part of the GALT, and play an essential role in the immune response to perturbations of the mucosal microenvironment [155].

Lymphoid follicles in the murine colon mucosa mostly localize in the lamina propria and sub mucosal region of the intestine and can be visualized by Haematoxylin-Eosin (H&E) staining of horizontal sections of the intestine, as dense aggregates of lymphocytes (**Figure 6A**). I then performed several specific stainings with antibodies directed to B220, CD3, CD11c and CD21, in order to highlight the heterogeneity and compartmentalization of lymphoid tissue. As expected, the majority of the cells composing TLT are B220+ B lymphocytes (Pink, **figure 6B**), while CD3+ T lymphocytes represent a minor component (light blue, **figure 6B**). CD11c+ dendritic cells constitute an important element of TLT (green, **figure 6C**). Another important population is represented by CD21+ FDC (green, **figure 6B/6D**), which play a key role in B cell recruitment, activation proliferation. In fact, they represent the major source of the lymphorganogenic chemokine CXCL13 (blue, **figure 6D**).

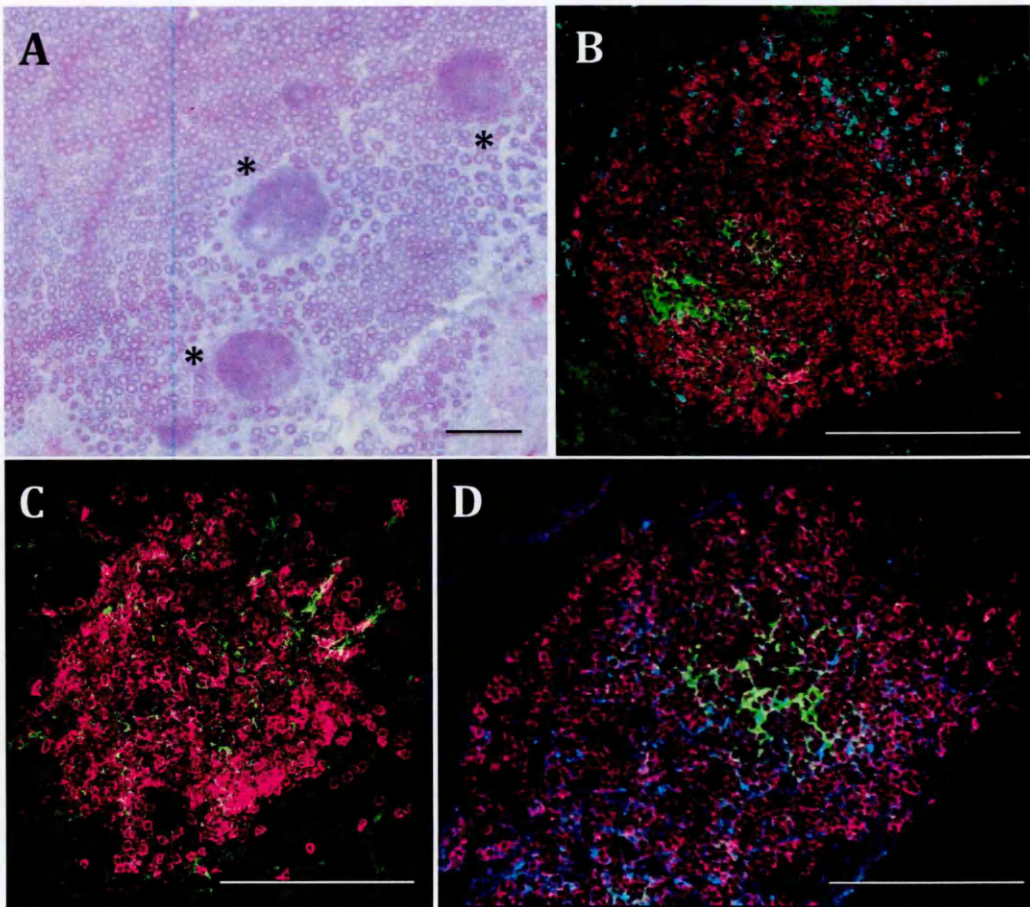


Figure 6. Characterization of TLT in the murine colonic mucosa. A representative image obtained by virtual scansion of a section of murine colon mucosa stained with Haematoxylin-Eosin. Asterisks indicate TLT (A). Staining with B220 antibody allows identification of TLT, since B cells are the major population. B (pink, B) and T cells (light blue, B) hold complementary positions within TLT. A core of FDCs (green, B) is present in the B cell area. CD11c+ DC (green, C,) are spread among B cells (pink, C). FDCs (green, D) are an important source of CXCL13 (Blue, D). Scale Bars: 200 μm (A), 50 μm (B-D)

Analysis of TLT in preclinical models of CRC

My previous analysis of TLT in human CRC suggests that a reactive stroma associated with carcinogenesis may provide proper signals inducing lymphoid neogenesis. To confirm the contribution of chronic inflammation to TLT formation, I evaluated its effect in two CRC mouse models:

- a mouse model of colitis-associated cancer (AOM/DSS)
- a genetic mouse model of CRC not driven by an inflammatory process (ApcMin/AOM).

In the first model, adenomas develop after AOM/DSS treatment due to the synergic action of the carcinogen (AOM) and the mucosal irritant (DSS) that causes chronic colitis (**Figure 7A**). In the second model, AOM treatment favours the formation of polyps in the distal colon of ApcMin mice that usually have polyps in the small intestine (**Figure 7B**).

In order to quantify TLT in the mucosa of tumour-bearing mice, I performed whole mount staining with an anti-B220 antibody, identifying B cells, which are the most represented population within TLT in the gut. Then I calculated the area of TLT by morphometric analysis. My results show that in the colonic mucosa of AOM/DSS treated mice there is a strong increase in the total area covered by B220+ TLT compared to control mice (**Figure 7C**); on the contrary, in ApcMin/AOM mice there is no a significant modification of lymphoid tissue area (**Figure 7D**), confirming the important role of inflammation in TLT development.

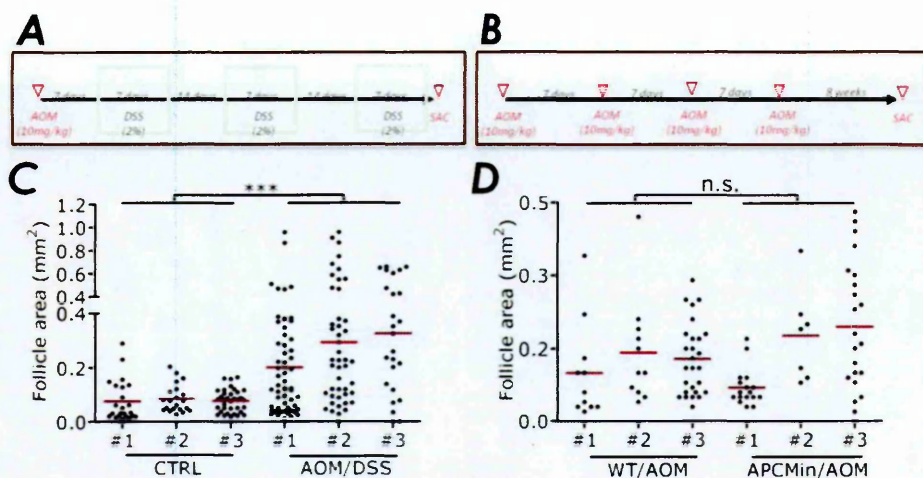


Figure 7. Chronic inflammation drives expansion of TLT in a preclinical model of CRC. Schematic protocol of AOM/DSS administration in the colitis-associated colorectal cancer model (**A**) and of AOM administration to ApcMin mice, in the genetic model of colorectal cancer not driven by an inflammatory reaction (**B**). At the end of the protocol, mice develop adenomas in the distal colon. The area of TLT was evaluated both in colon whole mounts of mice developing colonic adenomas and control mice. The area of TLT significantly increased after inflammation-induced polyp formation (**C**), while there was no significant modification of lymphoid tissue area in mice developing non-inflammation-driven adenomas (**D**). *** p < 0.001; Two-tailed p value by Student's t test.

TLT mediates T cell infiltration in a murine model of inflammation-driven CRC

I have previously discussed how TLT density positively correlates with CD3+ T cell density, supporting the idea that TLT is involved in T cell recruitment at the tumour site.

Since TLT increases in tumour-bearing mice compared to controls, I asked whether this expansion correlates with an increased amount of T cells reaching TLT. In order to answer this question, I quantified T cells by staining colon sections with an anti-CD3 antibody. Quantification revealed that control mice had very few

or none T cells within TLT (**Figure 8 A-B**), differently from tumour-bearing mice, which show a well defined T cell zone (**Figure 8C**).

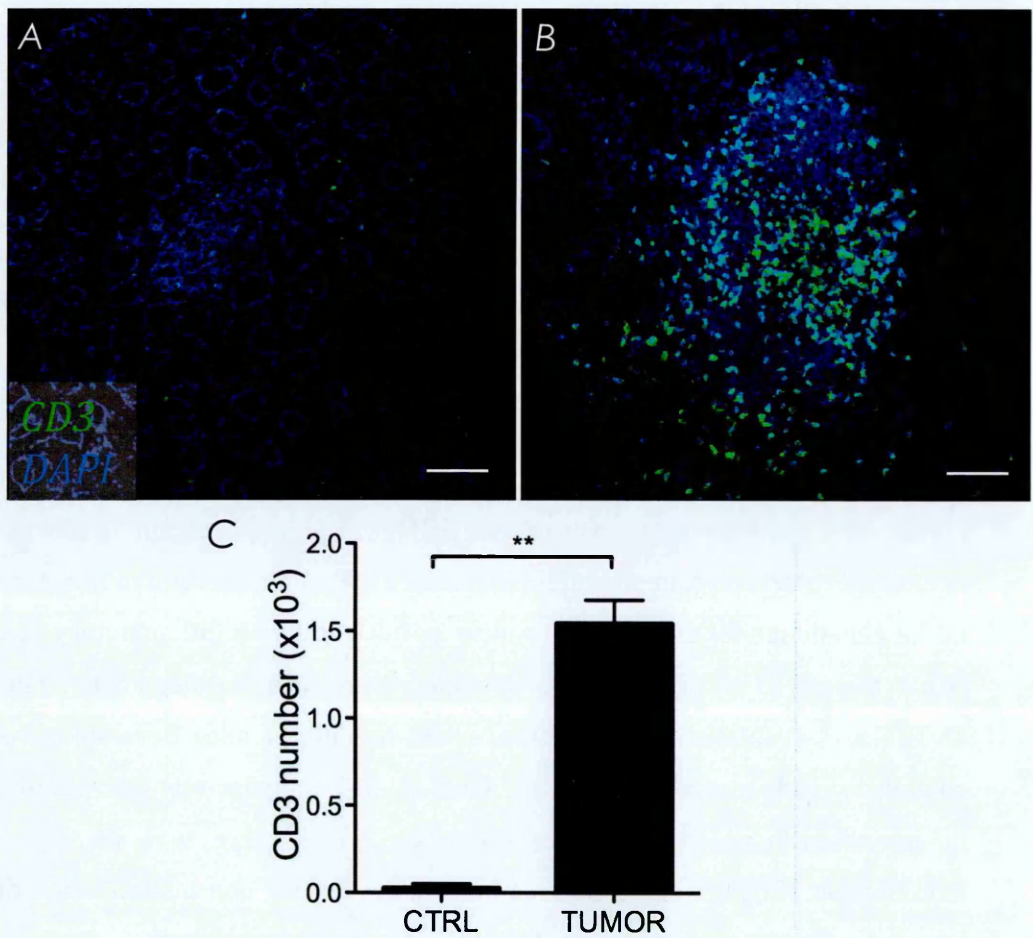


Figure 8. Expansion of TLT associates to increased number of CD3+ T cells. Staining with an anti-CD3 antibody allows the identification of T lymphocytes. In the colonic mucosa of control mice, TLT is almost completely devoid of T cells (**A**). On the contrary, a considerable number of T cells are present in tumour-associated TLT in AOM/DSS treated mice (**B**). There is a significant increase of CD3+ cell number in tumour-bearing mice compared to control mice (**C**). CTRL, n=6; AOM/DSS, n=6; scale bars: 200 μ m; ** p < 0.01 by Student's t test.

Analysis of the vessel network associated to TLT in a murine model of inflammation-driven CRC

As I highlighted above, the process of lymphoid neogenesis includes new vessel formation. In human CRC specimens, I have demonstrated the presence of both lymphatic vessels and HEVs, the latest increasing in tumour-associated TLT compared to TLT in the normal mucosa. In light of these previous results, I have analysed the vessel network surrounding and within TLT in AOM/DSS treated mice and controls. Staining with anti-CD31 antibody (**Figure 9A**), anti-Lyve1 (**Figure 9A**) and anti-PNAd (**Figure 9B**) revealed the presence of haematic and lymphatic vessels and specialized blood vessels, HEVs.

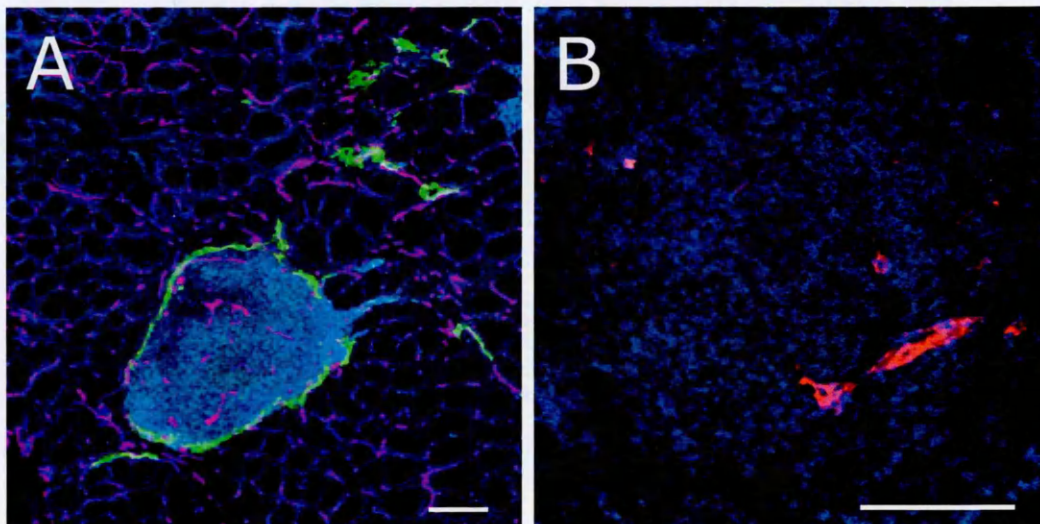


Figure 9. Characterization of the vessel network associated to TLT in the AOM/DSS model. Tumour-associated TLT contains CD31+ haematic vessels (pink, **A**) as well as Lyve1+ lymphatic vessels (green, **A**). PNAd+ HEV are present within TLT (orange, **B**). Scale bars: 200 μ m (**A**), 50 μ m (**B**)

Quantification of CD31+ lymphatic vessels in AOM/DSS murine model

To get insights into the mechanisms of vascular increase associated to TLT formation in colorectal cancer, I have quantified the vascular network surrounding TLT in the AOM/DSS model. In control mice, mucosal crypts are surrounded by a polygonal network of vessels and lymphoid tissue is drained by a regular vascular supply (**Figure 10A**). In contrast, TLT in the colon mucosa of tumour-bearing mice is surrounded by a dense network of haematic and lymphatic vessels (**Figure 10B**). Staining of colon whole mounts followed by morphometric tridimensional analysis of the lymphatic network allowed a precise evaluation and revealed a significant increase of lymphatic vessels draining TLT in mice with tumours compared to control mice (**Figure 10C**).

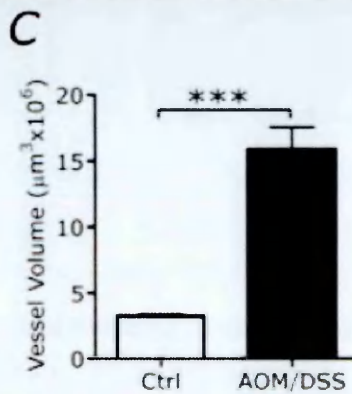
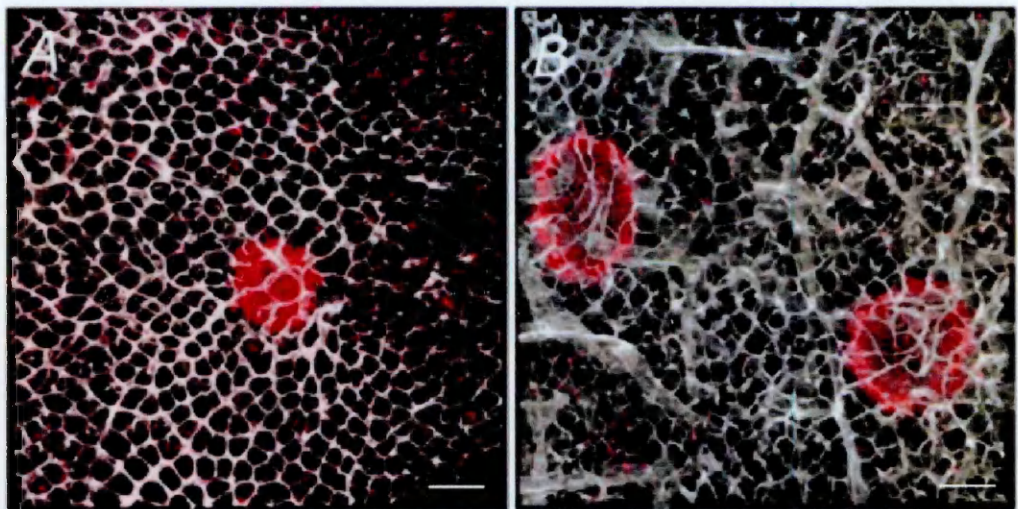
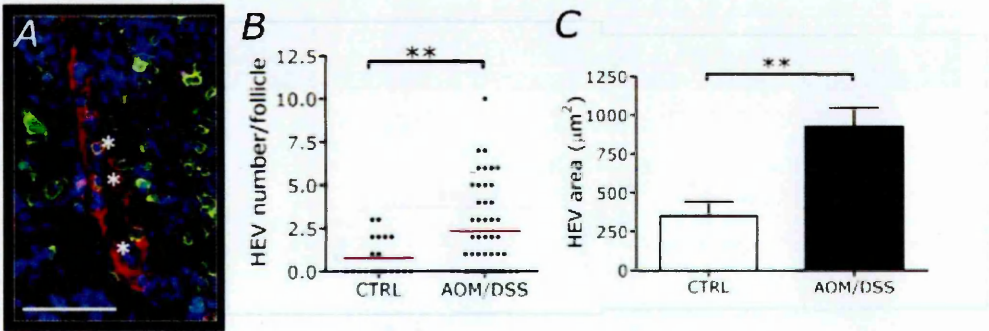


Figure 10. Quantification of the lymphatic network draining TLT in AOM/DSS murine model. The lymphatic network draining TLT in the colon mucosa of tumour-bearing mice (**B**) is expanded compared to control mice (**A**). Visualization of CD31+ vessels (grey in **A** and **B**) surrounding B220+ TLT (red). Lymphatic vessels were discriminated from CD31+ haematic vessels based on the morphology and the intensity of the staining. n=6 mice, CTRL; n=6 mice AOM/DSS; bars represent SEM. Scale bars: 200 μm . *** $p < 0.001$ by Student's t test.

Among haematic vessels, some expressed the markers MadCAM and PNAd, associated to HEVs (**Figure 9B-C**). In many sections, CD3+ T cells were adherent to HEVs, suggesting an active role of these vessels in lymphocyte recruitment (**Figure 11A**). Quantification of PNAd expressing haematic vessels revealed that tumour-associated TLT contained a higher number of PNAd+ HEVs, compared to TLT in the normal mucosa (**Figure 11B**), this accounting for an increase in the vascular area of these specialized vessels (**Figure 11C**).



Role of TLT in lymphocyte recruitment at the tumour site

Our data, both in CRC patients and in the AOM/DSS preclinical murine model of CRC, indicate that a high density of TLT correlates with a higher density of CD3+ T cells, suggesting that TLT is involved in lymphocyte recruitment. In order to demonstrate the contribution of TLT to lymphocyte recruitment at the tumour site, I injected intra venous GFP+ splenocytes both in controls and in AOM/DSS treated mice. After 24 hours, very few or none GFP+ cells localized within TLT associated to normal mucosa (**Figure 12A**); conversely, a considerable amount of GFP+ cells localized in tumour-associated TLT (**Figure 12B**).

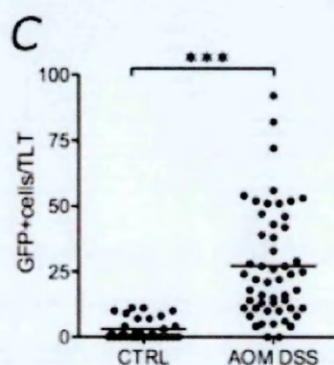
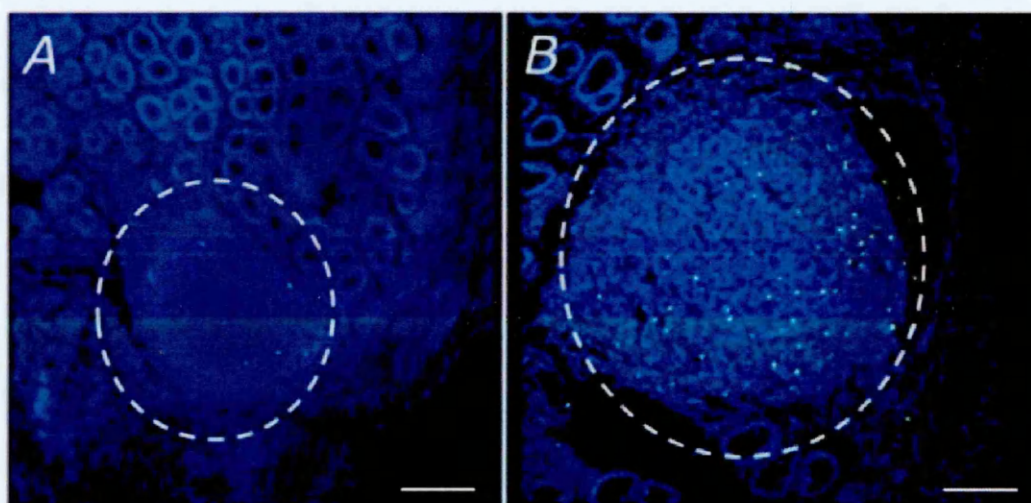


Figure 12. Transfer of GFP+ splenocytes into control and tumour-bearing mice. GFP+ splenocytes from wild-type mice were intravenously injected into AOM/DSS treated and control mice; mice were sacrificed after 24 hours from the injection and colons analysed by confocal microscopy. While TLT is almost devoid of GFP+ cells in colonic mucosa of control mice (A), a considerable number of GFP+ cells localized in TLT in the colonic mucosa of AOM/DSS treated mice (B). Quantification of GFP+ cells within TLT is shown, each dot refers to a single lymphoid follicle (C). Scale bars: 200 μ m; *** $p < 0.001$ by Student's t test

Clinical relevance of TLT in human CRC

The evaluation of CD3+ tumour-infiltrating lymphocytes (CD3-TILs) in the colonic mucosa of CRC patients has been object of intense studies, which have led to the remarkable conclusion that CD3-TILs contribute to an anti-tumour immune response and are associated to a favourable prognosis in CRC patients.

Once established that there is a close relationship between CD3-TLT and CD3-TILs, the next step aimed to understand the clinical significance of CD3-TLT. **Table 1** summarises clinico-pathologic features of patients included in our cohort.

CD3-TLT was present in 276 (78,6%) of 351 CRC specimens, with a density median value of 2,68%. In order to investigate the clinical relevance, I correlated CD3-TLT density with clinical parameters and follow-up (disease-free survival, DFS). Univariate analysis showed that high (>median) CD3-TLT and CD3-TIL density significantly correlated with better outcome [HR, 0.62; 95% confidence interval (CI), 0.40–0.97]. However, Cox multivariate analysis considering all the variables revealed an interaction of CD3-TLT and CD3-TILs with nodal status in predicting patients relapse, which suggests that the ability of CD3-TLT and CD3-TILs to predict patients relapse may change according to nodal status (**Table 2**).

		n	(%)
Patient Age ^a			
≤69 yrs		187	53%
>69 yrs		164	47%
Patient Gender			
Male		196	56%
Female		155	44%
Tumor Site			
Colon		263	75%
Rectum		88	25%
Tumor Invasion ^c			
pT3		311	89%
pT4		40	11%
Nodal Status ^d			
N0		185	53%
N1		106	30%
N2		60	17%
Tumor Grade ^e			
G1/G2		288	82%
G3		63	18%
Tumor Cell Type ^f			
Adenocarcinoma		319	91%
Variants		32	9%
Vascular Invasion			
No		273	78%
Yes		78	22%
MS-Status ^g			
MSS		291	83%
MSI		60	17%
5-FU Treatment			
Stage II	No	108	58%
	Yes	77	42%
Stage III	No	48	29%
	Yes	118	71%

Table 1. Demographics and clinico-pathologic features of 351 CRC.

^a Age entered as a continuous variable. ^c: pT3: invading through the muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues. pT4: directly invading adjacent organs or perforating visceral peritoneum. ^d: N0: no lymph node involvement. N1: 1-3 nodes involved. N2: more than or equal to 4 nodes involved. ^e: G1: well differentiated. G2: moderately differentiated. G3: poorly differentiated. ^f: Variants mucinous or medullary. ^g: MSI: microsatellite-unstable. MSS: microsatellite-stable.

	Relapse		Univariate Analysis		Multivariate Analysis	
	No (n=267)	Yes (n=84)	HR (95% CI)	p	HR (95% CI)	p
TLT density						
<Median	125	51	1.00 (reference)		1.00 (reference)	
≥Median	142	33	0.62 (0.40-0.97)	0.03	0.72 (0.46-1.14)	α
TIL density						
<Median	136	50	1.00 (reference)		1.00 (reference)	
≥Median	131	34	0.74 (0.48-1.15)	0.18	0.83(0.53-1.30)	β
Age (years, Std Dev) ^a	(66.94, 11.45)		1.00 (0.98-1.02)	0.64 ^a		
Gender						
Male	145	51	1.00 (reference)			
Female	122	33	0.81 (0.52-1.26)	0.35		
Tumor MS Status ^g						
MSS	215	76	1.00 (reference)			
MSI	52	8	0.56 (0.27-1.16)	0.12		
Tumor Site						
Colon	214	49	1.00 (reference)		1.00 (reference)	
Rectum	53	35	2.24 (1.45-3.46)	<0.001	1.87 (1.20-2.92)	0.006
Local Invasion ^c						
pT3	242	69	1.00 (reference)			
pT4	25	15	1.95 (1.11-3.42)	0.01		
Tumor Nodal Status ^d						
N0	159	26	1.00 (reference).		1.00 (reference)	
N1	78	28	1.99 (1.16-3.39)	0.01	1.98 (1.16-3.39)	α, β
N2	30	30	4.44 (2.62-7.53)	<0.001	3.75 (2.19-6.41)	

Tumor Grade ^e					
G1/G2		222	66	1.00 (reference).	
G3		45	18	1.33 (0.79-2.24)	0.28
Tumor Cell Type ^f					
Adenocarcinoma		245	74	1.00 (reference)	
Variants		22	10	1.42 (0.73-2.75)	0.29
Tumor Vascular Invasion					
No		214	59	1.00 (reference)	
Yes		53	25	1.64 (1.03-2.63)	0.03
5-FU Treatment					
Stage II	No	108	17	1.00 (reference)	
	Yes	77	14	1.36 (0.63-2.94)	0.42
Stage III	No	36	26	1.00 (reference)	
	Yes	98	48	0.65 (0.38-1.15)	0.14

Table 2. Univariate and Multivariate Cox hazard model for predictive factors of disease relapse in 351 pT3/pT4 CRC.

a: Age entered as a continuous variable. α , and β : the density of TLTs (\geq median; α) and the density of TILs (\geq median; β) are interacting with the occurrence of nodal metastasis in predicting patient's relapse (P=0.07 and P=0.03 respectively). Cox proportional hazard model: Hazard ratio (HR) less than 1.00 represents a decreased likelihood of patients' relapse, whereas a HR greater than 1.00 represents an increased likelihood. Multivariate analysis was performed by entering only variables with a P value less than 0.20 at Univariate analysis. By a backward stepwise elimination approach, non-significant variables, and their non-significant interactions, were removed from the model.

^c: pT3: invading through the muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues. pT4: directly invading adjacent organs or perforating visceral peritoneum. ^d: N0: no lymph node involvement. N1: 1-3 nodes involved. N2: more than or equal to 4 nodes involved. ^e: G1: well differentiated. G2: moderately differentiated. G3: poorly differentiated. ^f: Variants mucinous or medullary. ^g: MSI: microsatellite-unstable. MSS: microsatellite-stable.

To dissect better this aspect, I performed subgroup analysis, evaluating the prognostic function of CD3-TLT and CD3-TILs in subgroups of patients, according to their nodal status. Subgroup analysis revealed that in patients with node-negative CRC (Stage II; n=185), a high density of CD3-TLT (>median, 2.68%) and of CD3-TILs (>median, 2.06%) was associated with better prognosis compared to patients with a low density of CD3-TLT and CD3-TILs (log-rank test, $p = 0.02$ in both cases) (**Figure 13A-B, left**). Conversely, CD3-TLT and CD3-TIL densities were irrelevant to predict the prognosis of patients with node-positive CRC (Stage III; n=166) (log-rank test, $p = 0.46$ and $p = 0.64$, respectively) (**Figure 13 A-B, right**), thus behaving as prognostic biomarkers only in early stage CRC.

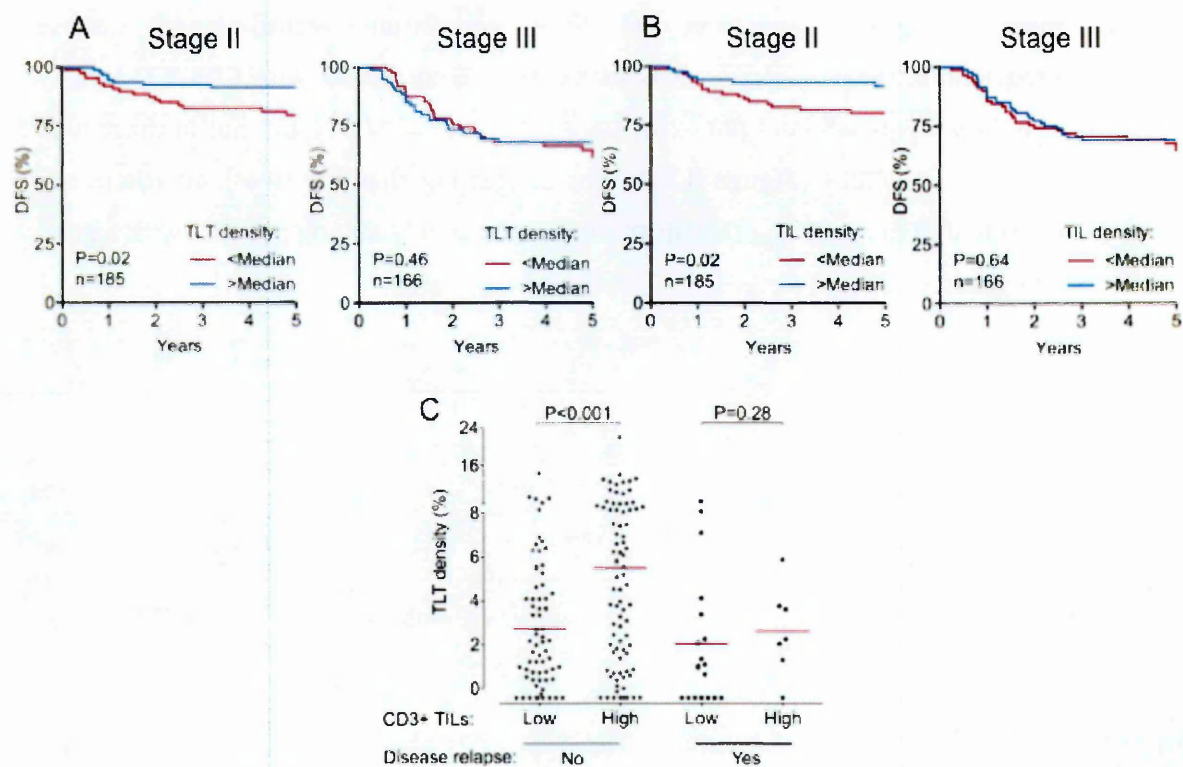


Figure 13. Clinical relevance of tumour-associated TLT and TILs in 351 CRC patients. (A-B) Kaplan-Meier curves showing disease-free survival (DFS), according to TLT and TIL density. A high density of TLT (\geq median, 2.68%) and of TILs (\geq median, 2.06%) is associated with better outcome in patients with node-negative CRC ($p = 0.02$, $n=185$) (A-B, *left*), but not in those with node-positive CRC ($p = 0.46$ and $p = 0.64$ respectively, $n=166$) (A-B, *right*). (C) Coordination of TLT and TIL immune infiltration. Distribution of TLT IRA% according to the CD3+ density (\geq median) in relapsing and not relapsing stage II CRC patients. TLT and TILs correlate only among patients that do not relapse.

We then hypothesized that CD3-TLT and CD3-TILs have a comparable prognostic impact and to better understand their relationship in the tumour microenvironment, we analysed their correlation according to disease progression. What we found was that CD3-TLT correlates with CD3-TIL density only in patients who did not experience relapse ($p = 0.001$), but not in those who relapsed ($p = 0.28$) (**Figure 13C**), thus suggesting that the two biomarkers are coordinated in mediating the antitumor response only among patients with a good prognosis.

Discussion

In this first part, I presented data on the characterization and the clinical relevance of tertiary lymphoid tissue in colorectal cancer.

From the first immunohistochemical analysis of human colorectal cancer specimens, I observed that CD3+ tumour-infiltrating lymphocytes are present both interspersed within the tumour tissue and in the stroma and they also aggregate in lymphoid structures, showing features of TLT. One of the main characteristics of TLT is the peculiar structural organization, with distinct cell compartments and specialized cell subsets, interacting each other to maintain a potentially functional environment.

Specifically, further immunohistochemical analysis with specific antibodies highlighted the compartmentalization of tumour-associated TLT, where CD3+ T cells and CD20+ B lymphocytes hold complementary positions, with several classes of dendritic cells among them. DC-Lamp+ mature dendritic cells are present scattered throughout lymphoid tissue and CD21+ follicular dendritic cells constitute a core within B cell zone.

In a physiologic context, lymphoid organs orchestrate lymphocyte trafficking, guiding them through a network of lymphatic and specialized haematic vessels. Interestingly, analysing the vascular network associated to TLT, I found both Lyve+ lymphatic vessels and PNAd+ specialized haematic vessels, HEVs. The presence of HEVs suggests a role for TLT in T cell recruitment at tumour site, while lymphatics suggest a possible active process of lymphocyte migration in and out the tumour site. Importantly, HEVs were present primarily in the context of TLT and very rarely in the surrounding tumour tissue. Analysing this specific vascular network within TLT, I found an increase in HEV number in intra-tumour TLT compared to ILF associated to the normal mucosa. This finding points out *de novo* formation of tumour-associated TLT, during carcinogenesis, and, since HEVs are used to direct the traffic of naïve T cells, suggests the possible active role of TLT in lymphocytes recruitment at tumour site. Thus, the idea is that the more TLT with

HEVs is present in the tumour context, the more tumour-infiltrating lymphocytes can reach the tumour. This process of lymphoangiogenesis is mediated by interactions between the vascular endothelium and immune cells, among which dendritic cells seem to have a pivotal role [156, 157]. Concerning haematic vessels, in several solid tumours TLT-associated HEVs mediate T cell recruitment and correlate with a better clinical outcome.

In order to test the hypothesis and to find a relationship between TLT and TILs, I performed a whole tissue analysis of CD3+ infiltrate on CRC patient sections. This analysis showed a positive correlation between CD3+ T cell density and TLT density. Patients with a high density of CD3-TLT also had a high density of CD3-TILs infiltrating the tumour. The mechanism of the correlation between CD3-TLT and CD3-TILs can be explained by the increase in HEV network in tumour-associated TLT. Thus, the vessel expansion in tumour associated TLT suggests a possible key role of TLT in the recruitment of CD3+ T cells to the tumour site.

I then investigated the clinical significance of CD3-TLT in relationship with CD3-TILs in a large cohort of CRC patients. The most important finding was that a high density of CD3-TLT and CD3-TILs associate to a better prognosis only in patients with node-negative CRC, while in patients with node-positive CRC CD3-TLT and CD3-TIL density are irrelevant to predict the prognosis. These results highlight the similar prognostic behaviour of CD3-TLT and CD3-TILs, confirming their relationship in the tumour microenvironment. Importantly, CD3-TLT and CD3-TILs are independent prognostic markers for better prognosis in stage II CRC and their prognostic relevance is comparable.

The presence of specific subsets of cells within TLT has been investigated as key determinant of the anti-tumour function of TLT. For instance, it has been demonstrated that the positivity for the markers DC-Lamp or CD20 within TLT is predictive of prognosis in lung cancer and melanoma [54, 127, 152]. Significantly, in lung cancer the prognostic value of DC-Lamp+ cells within TLT was significantly

independent of CD8⁺ T cells, and when matched as immune signature, their prognostic relevance was highly increased [158]. Consequently, TILs were likely dependent from DC-Lamp⁺ TLT to exert their anti-tumour activity and to facilitate the identification of lung cancer patients with better prognosis.

The accurate compartmentalization of TLT presumes the presence and involvement of specific molecular mediators released in the tumour microenvironment. The developmental process of TLT resembles the physiological process of SLO formation, but it occurs in the inflamed areas of several pathological conditions [115, 117, 159]. In this context, the triggering event is the reactivation of stromal cells in local tissues by inflammatory mediators, such as lymphoid chemokines [160]. The presence of TLT that has been reported in several tumour types is in line with the relationship between chronic inflammation and tumour development, as the inflamed tumour microenvironment favours TLT development. In chronic inflammatory conditions, activated stromal cells are known to produce several inflammatory mediators, including chemokines, responsible for the infiltration of immune cells. The hypothesis that the chronic inflammatory response associated to tumorigenesis may be the trigger for TLT development has been confirmed by staining collagen fibres (Sirius Red) and activated fibroblasts (alpha-SMA), revealing the presence of a reactive stroma surrounding tumour-associated TLT, suggestive of an inflammatory microenvironment. Moreover, the expression of CXCL13 and CCL21 by α -SMA mesenchymal cells suggested an active recruitment of B and T cells and plasticity of TLT. Thus, the stromal reaction in colorectal cancer is likely to drive the formation of intra-tumour lymphoid tissue organized as TLT.

Besides the highly organized microarchitecture and the complexity of the cellular and molecular network, an important point that could help understanding the mechanism of TLT formation is the localization of TLT in the mucosa of colorectal cancer patients. Interestingly, TLT is located at the invasive front of the tumour, within the stroma. Its special location helps in summarizing three important points:

- the presence of TLT within the stroma confirms the important contribution of stromal cells to the formation of intra-tumour lymphoid tissue;
- intra-tumour TLT has a distinct localization compared to lymphoid tissue associated to the normal mucosa (under the crypts). This allows to argue that tumour-associated TLT formation takes place during tumour development, thus representing a process of lymphoid neogenesis;
- TLT is located at the invasive front and this may account for its involvement in the anti-tumour immune response.

At this point, preclinical murine models have helped me to better clarify and confirm the role of chronic inflammation in TLT formation and the association between TLT and TILs.

As in humans, in murine colon mucosa ILFs are already present in homeostatic conditions, as part of the gut-associated lymphoid tissue and are located in the lamina propria of the colon. Immunofluorescence analysis pointed out the heterogeneity and compartmentalization of ILFs, with a prevalent B cell population, few T lymphocytes and CD11c+ dendritic cells. Importantly, a core of CD21+ FDCs is present within B cell zone; these DCs play a key role in B cell recruitment, with CXCL13 production, activation and proliferation. Since in biological science, in most cases structure is related to function and function strictly depends on structure, the highly organisation of murine ILFs, as in humans, suggests they should have important roles during individual life. First of all, I demonstrated that in a murine model of inflammation-driven CRC, TLT expands in tumour-bearing mice compared to controls, while in a murine model of CRC not directly related to inflammation I didn't find any difference between control and tumour-bearing mice. This confirms the important role that chronic inflammation has in TLT development.

Then I analysed TLT behaviour in the inflammation-driven preclinical model. Going deeper into TLT function, I found that the number of CD3+ T cells within TLT is higher in tumour-bearing mice, differently from controls that have very few or none T cells, as I have previously demonstrated in CRC-patients. When TLT expands, there is an increased amount of T cells reaching TLT. Asking how these T cells reach lymphoid tissue, I have shown that tumour-associated TLT is well vascularized by CD31+ haematic vessels and Lyve1+ lymphatics. Among CD31+ haematics that increase during tumorigenesis in the surrounding tissue of TLT HEVs play the principal role in mediating T cell recruitment. Indeed, I observed a strong increase of these specialized haematic vessel, that correlates with the increased T cell infiltration in tumour-bearing mice. The final evidence that TLT is involved in lymphocyte recruitment at the tumour site came from an experiment of cell transfer. GFP+ splenocyte i.v. injection showed that a considerable amount of these cells reached tumour-associated TLT.

Therefore, TLT is associated with lymphocyte infiltration in CRC, being involved in TIL recruitment. CD3-TLT and CD3-TILs work together to set up an anti-tumour immune response in patients with low-risk early-stage colorectal cancer. Thus, CD3-TLT represents a novel prognostic biomarker for CRC.

PART II

B CELL
DISTRIBUTION
IN
COLORECTAL
CANCER

Background and goal

This part of my thesis will focus on a key population among immune cells infiltrating colorectal tumours: B cells. In the introduction, I have discussed about the duality of B cell role in cancer and how the impact of B cells on the clinical outcome of cancer patients is debated. On one hand, B cells can produce antibodies or act as APCs, promoting the anti-tumour activity of tumour-infiltrating T cells [77, 78, 161]; from the other, the production of antibodies and cytokines can foster tumour development in a paracrine fashion [81, 82, 84]. In addition, subsets of B cells with regulatory function have been recognized and involved in cancer progression [72, 73]. Collectively, despite still controversial, increasing evidence that B cells play a role in cancer progression has been provided [162], bringing up the hypothesis that also B-cell responses should be considered as targets of immunotherapeutic approaches. I have already shown the presence of B cells within TLT in human colorectal cancer patients and in preclinical models of CRC. The next step was to gain insights into the role of B cells in colorectal tumours and investigate whether they have a prognostic significance.

B cells in human colorectal cancer

B cells in human colorectal cancer

In the previous part of the results I focused my attention on tumour infiltrating CD3+ T cells, showing their peculiar distribution within tumour tissue. In a similar manner, focusing on B cells, I stained tissue slides from human CRC specimens with an anti-CD20 antibody. As shown in **Figure 14**, CD20+ tumour-infiltrating B cells localized both in tertiary lymphoid tissue in the stromal compartment (CD20-TLT) (**Figure 14A**) and scattered around the tumour margin (CD20-TILs) (**Figure 14B**).

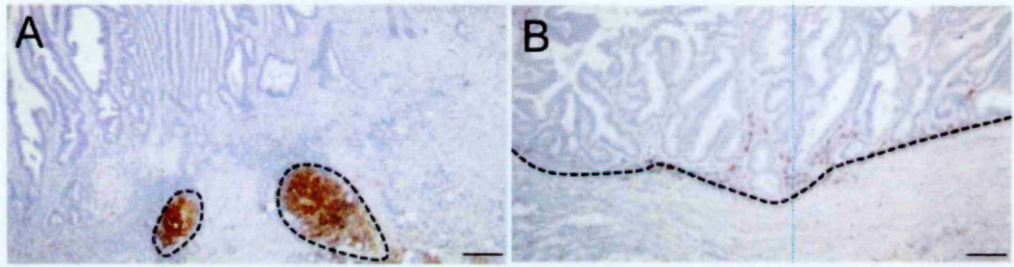


Figure 14. Distribution of B cells in the colonic mucosa of colorectal cancer patients. Representative images obtained by virtual scansion of a colorectal tumour specimen. Staining with anti-CD20 antibody shows that B cells distribute both within TLT (CD20-TLT, dotted circle) in the tumour stroma at the invasive front (A) and scattered around the tumour margin (CD20-TILs, dotted line) (B). Counterstaining with haematoxylin. Scale bars: 500 µm.

Antibody secreting cells (ASC), i.e. activated B cells, are abundantly present in the normal colon mucosa, most of them producing IgA. In order to investigate whether the physiological function of B cells changes in tumour conditions, I analysed their immunoglobulin profile. Staining with anti-IgA and anti-IgG antibodies revealed that the majority of B cells infiltrating the normal colon mucosa are IgA+ (**Figure 15 A**), consistent with the repertoire of B cells in the secretory mucosal epithelium during homeostasis. On the contrary, very few or none tumour infiltrating B cells, including CD20-TLT and CD20-TILs were IgA+ (**Figure 15B**); they appeared predominantly as IgG+ cells (**Figure 15C**), suggesting a different antigenic response compared to normal colonic mucosa. In this regard, recent works have shown an important immunosuppressive function for IgA expressing B cells [73], while tumour-antigen specific immunoglobulins can be crucial triggers of anticancer cellular immune responses [161].

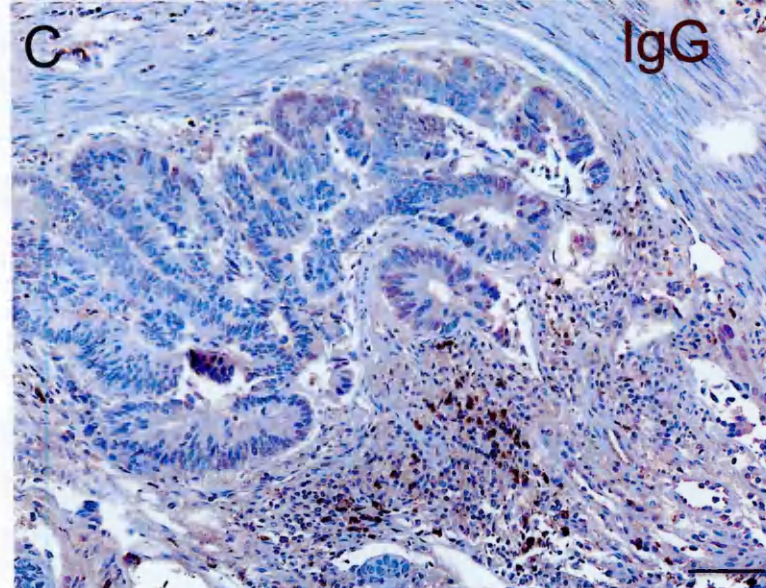
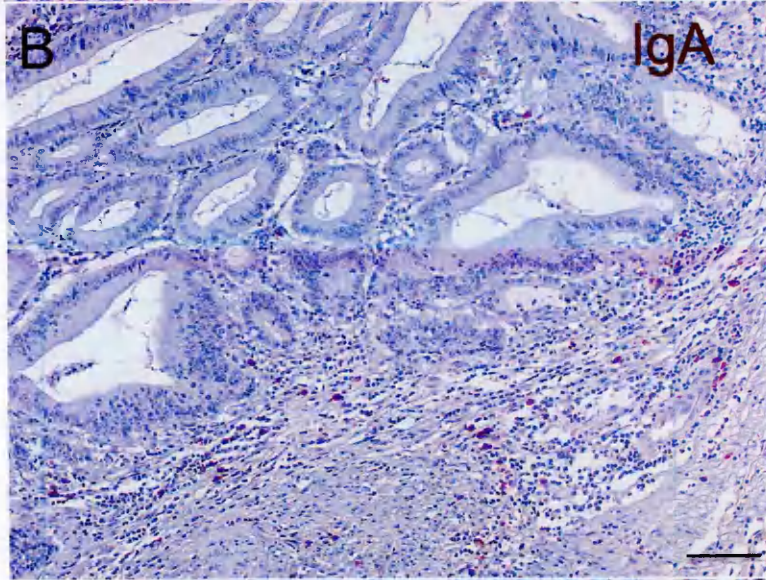
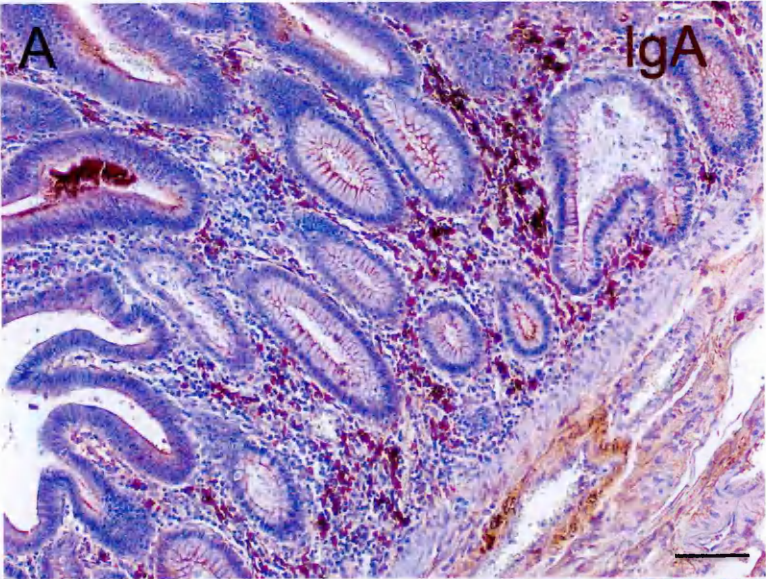


Figure 15. Immunoglobulin expression on tumour-infiltrating B cells. Immunohistochemistry with anti-IgA (A-B) and anti-IgG (C) antibodies on human colorectal cancer slides. In the normal colonic mucosa, as appears in A, most of B cells in the lamina propria are IgA+. In contrast, in tumour tissue, there are few IgA+ B cells (B) and there is a high prevalence of IgG+ tumour-infiltrating B cells, including B cells within TLT (C). Counterstaining with haematoxylin. Scale bars: 500 µm.

The presence of a germinal centre within the B cell area of lymphoid tissues is mandatory for the development of both high-affinity class-switched antibodies and memory humoral immune response. With the aim to understand if CD20-TLT could be site for organizing a humoral immune response, I assessed by immunohistochemistry if B cells express two markers of activated germinal centres. Staining with anti-Ki-67 (**Figure 16A**), indicating cell proliferation, revealed a group of cells that strongly expressed this marker within TLT. Moreover, staining with anti-BCL-6 (**Figure 16B**), a transcriptional factor that regulates a program of genes required for GC function, showed some positive cells, thus confirming the presence of active germinal centres within TLT. Overall, the profile of the B cell compartment of TLT suggests that this immune site is fully equipped to sustain B cell activation in human CRC.

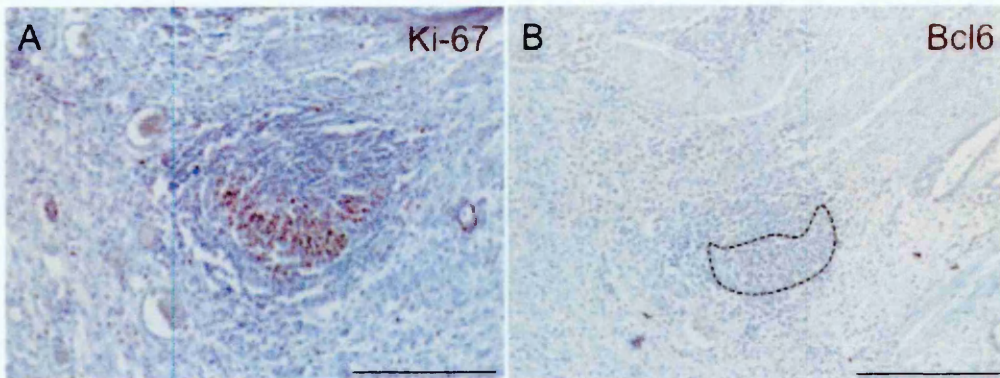


Figure 16. Expression of germinal centre markers in tumour-associated CD20-TLT. Immunohistochemistry with anti- Ki-67 (A) and anti- Bcl6 (B) antibodies on human colorectal cancer consecutive slides. In figure A, a Ki-67 core of cells is present in TLT. In figure B, some positive BCL-6 cells are present in the same area where Ki-67+ cells localized. Scale bars: 100 μ m (A), 200 μ m (B)

Prognostic relevance of B cells in human colorectal cancer

Once established how B cells localize in the tumour context, I asked whether the two distinct components differently contribute to the immune microenvironment in human CRC. In order to answer this question, I systematically and quantitatively evaluated the percentage of immune reactive area (IRA%) of CD20+ cells at the tumour invasive margin, by considering CD20-TLT and CD20-TILs as distinct immune populations. This analysis has been done on 204 tissue specimens from stage III CRC patients (**Table 3**); thus, compared to the previous analysis performed on T cells, we only considered Stage III patients. Whole tissue analysis highlights that CD20+ cells preferentially located within TLT compared to interspersed CD20-TILs (**Figure 14A-B**). Indeed, the density of B cells in TLT is significantly higher compared to the density of scattered CD20-TILs (**Figure 17A**), suggesting that B cells within TLT represent a predominant population compared to scattered CD20-TILs cells.

Next step was to investigate the clinical relevance of both CD20-TLT and CD20-TILs in a retrospective cohort study. To test the prognostic independency of immune biomarkers, we performed a Cox multivariate analysis, with respect to

other demographics, clinical, histo-pathologic features in colorectal cancer (**Table 4**). Multivariate analysis showed that CD20-TLT associated with better prognosis independently by any other variable included in the model ($p=0.01$), while CD20-TILs did not (**Table 4**). This result indicates that only TLT accounts for the prognostic relevance of B cells in human CRC, suggesting that the influence of B cells on tumour progression differs whether they are localized within lymphoid tissue or are interspersed at the tumour-host interface.

To gain further insights in the different prognostic behaviour of B cells and the relationship between CD20-TLT and CD20-TILs, we analysed the linear regression among the density of CD20-TLT and CD20-TILs according to CRC patient's disease progression. Multiple linear regression analysis revealed that the density of CD20-TLT independently correlated with the density of CD20-TILs ($r=0.16$; $p=0.02$, $N=204$), consistent with the ability of TLT to mediate recruitment of B lymphocytes. However, CD20-TILs correlated with CD20-TLT only among patients who experienced tumour recurrence, ($r=0.40$, $p<0.001$, $N=71$) (**Figure 17B**), while the correlation was lost among CRC patients with good prognosis ($r=0.10$, $p=0.21$, $N=133$) (**Figure 17C**). Thus, in sharp contrast to the relationship of CD3-TLT with CD3-TILs I have shown in the previous chapter, the correlation between CD20-TLT and interspersed CD20-TILs occurs only in patients who experienced recurrence of disease, suggesting that recruitment of CD20-TILs might reflect pro-tumour inflammatory pathways and that in the CRC microenvironment distinct populations of B cells critically regulate tumour progression.

Table 3. Patient demographics and tumour features according to densities of CD20-TLT and TILs and CD3-TILs in 204 stage III CRC.

		Immune Reactive Area (IRA%) ^{a,b}							
				CD20-TLT		CD20-TILs		CD3-TILs	
		n	(%)	Median	P	Median	P	Median	P
All cases		204	(100)	2.01		0.17			
Patient demographics									
Age	< 50 yrs.	15	(7.0)	2.51		0.11		8.33	
	50-70 yrs.	102	(50.0)	2.23	0.39	0.21	0.90	1.99	<0.001
	≥ 70 yrs.	87	(42.6)	1.78	0.10	0.21	0.25	2.15	<0.001
Gender	Female	128	(62.7)	1.89		0.21		2.39	
	Male	76	(37.2)	2.04	0.98	0.09	0.09	1.99	0.51
Tumour features									
Nodal	N1	132	(64.7)	2.20		0.19		2.33	
Invasion	N2	72	(35.3)	1.50	0.06	0.14	0.66	2.07	0.02
Angio	pT3	141	(69.1)	2.20		0.21		2.00	
Invasion	pT4	63	(30.9)	1.40	0.20	0.10	0.04	2.37	0.58
Local	pT3	173	(88.4)	2.01		0.17		2.24	
Invasion	pT4	31	(11.6)	1.86	0.99	0.16	0.75	2.01	0.79
Grade ^c	G1/G2	154	(84.8)	2.01		0.17		2.18	
	G3/G4	50	(25.5)	1.86	0.52	0.18	0.76	2.53	0.58
Tumor type	ADC	179	(87.7)	2.04		0.18		2.24	
	Mucinous ^d	25	(12.2)	1.23	0.35	0.08	0.18	1.79	0.92
	Dx	73	(35.8)	2.36		0.21		3.36	
Site	Sx	74	(36.3)	1.80	0.27	0.18	0.91	1.99	0.05
	Rectum	57	(27.9)	1.74	0.15	0.08	0.71	1.74	0.08
Chemotherapy (CTX)									
Adjuvant	No	62	(30.4)	1.50		0.11		2.04	

therapy	Yes	142	(69.6)	2.17	0.26	0.18	0.14	2.23	0.20
Immune infiltration									
CD20-TLT (IRA%)^a	< Median	103	(50.5)			0.10		1.64	
	> Median	101	(49.9)	NA	NA	0.28	0.02 ^f	3.06	0.001 ^f
CD20-TILs (IRA%)^a	< Median	102	(50.0)	1.31				1.93	
	> Median	102	(50.0)	2.80	0.02 ^f	NA	NA	2.87	0.10 ^f
CD3-TILs (IRA%)^a	< Median	103	(50.5)	1.65		0.15			
	> Median	101	(49.9)	2.42	0.00	0.21	0.10 ^f	NA	NA

^a Density is expressed as percent immunoreactive area at the tumour invasive margin. ^b linear regression analysis; CD20-TLT and TILs and CD3-TIL densities were entered as dependent continuous variables. ^c G1/G2, well-to moderately differentiated; G3/G4, poorly differentiated. ^d ADC: adenocarcinoma. ^e mucinous, signet-ring, or medullary histology. ^f multiple linear regression analysis, immune variables were entered together in the model. NA not applicable.

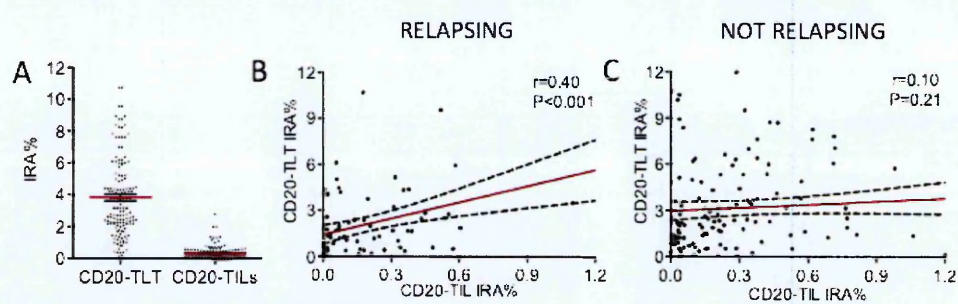


Figure 17. Distribution of B cells in spatially distinct compartments and their correlation in human CRC. (A-C) Density of B cells in TLT (CD20-TLT) is significantly higher compared to density of scattered CD20-TILs, highlighting how B cells preferentially localize within tertiary lymphoid tissue in human CRC. ** p < 0.01 value by Student’s t test (A). Linear regression between the distributions of CD20-TLT and of CD20-TILs according to CRC patient’s disease progression. CD20-TLT and CD20-TILs correlate only among patients who relapse (B) (p <0.001; r=0.4; n=71), while they do not correlate in patients who do not relapse (p <0.21; r=0.1; n=133) (C); p value by Pearson simple linear regression analysis.

Table 4. Predictors of post-surgical disease specific recurrences in 204 patients with nodal invasive pT3/pT4 colorectal cancer patients.

		Univariate		Multivariate	
		Analysis ^a		Analysis ^a	
	Recurrence Rate (%)	H.R. (95%C.I.)	P	H.R. (95%C.I.)	P
CD20-TLT ^b	<	1.00 ref.		1.00 ref.	
	>	0.44 (0.27-	0.001	0.54 (0.33	0.01
	Median	0.73)		0.89)	
CD20-TILs ^b	<	1.00 ref.			
	>	0.67 (0.42-	0.10		
CD3-TILs ^b	<	1.00 ref.			
	>	0.98 (0.62-	0.94		
Age	< 50	1.00 ref.			
	50-70	0.71 (0.27-	0.48		
	≥ 70	1.27 (0.50-	0.62		
Gender	Female	1.00 ref.			
	Male	1.11 (0.69-	0.66		
Nodal invasion	N1	1.00 ref.		1.00 ref.	
	N2	2.74 (1.71-	<0.001	2.35 (1.46-	<0.001
		4.38)		3.77)	1
Angio invasion	No	1.00 ref.		1.00 ref.	
	Yes	2.42 (1.51-	<0.001	1.96 (1.22-	0.005
		3.86)		3.15)	
Tumor cell type	ADC	1.00 ref.			
	Variant	1.26 (0.64-	0.50		
Local invasion	pT3	1.00 ref.			
	pT4	1.32 (0.72-	0.36		
Grade ^c	G1/G2	1.00 ref.			
	G3	1.63 (0.98-	0.06		
Tumor site	Dx	1.00 ref.			
	Sx	0.70 (0.39-	0.24		
	Rectum	1.31 (0.75-	0.34		
MS-Status	MSS	1.00 ref.			
	MSI	0.78 (0.34-	0.57		
Adjuvant treatment	No	1.00 ref.			
	Yes	0.61 (0.38-	0.05		

^a COX regression analysis, ^b Densities as percent of immunoreactive area at the tumour invasive margin.

^c G1/G2, well-to moderately differentiated; G3, poorly differentiated.

* By a backward stepwise elimination approach, non-significant variables ($P < 0.20$) were removed from the multivariate model.

B cells in murine colorectal carcinogenesis

B cells in murine colorectal carcinogenesis: the AOM/DSS model

To expand our knowledge on the role of B cells in colorectal cancer and dissect the duality of the B-TLT and B-TIL immune components, I took advantage of two CRC preclinical models showing a different distribution of B cells in the microenvironment: the AOM/DSS model, characterized by well-defined TLT [53] and the implantable CMT93 model, which doesn't show organised structures, likely due to the rapid tumour growth (21 days) and absence of chronic inflammatory reaction. I also took advantage of mice lacking B cells (μ MT), to investigate the contribution of B cells in the tumour microenvironment.

As a first step, I evaluated by flow-cytometry the number of B220+ cells in the colon mucosa of AOM/DSS treated mice and controls. To this aim, I isolated leukocytes from the lamina propria (LPL) and stained them with different antibody cocktails (**Figure 18-19**). Flow cytometry quantitative detection of immune cells in the tumour colon mucosa of AOM/DSS treated mice revealed a massive infiltrate of CD19+/B220+B cells as compared to controls (**Figure 18A-B**). A further analysis of B cells by immunofluorescence with anti-B220 antibody revealed that B cell distribution in the murine colon mucosa is similar to that found in human CRC specimens, with B220+ B cells localized in two distinct compartments, either scattered in the stroma of tumour tissue as interspersed cells (B220-TILs; dot line) or as a component of tertiary lymphoid tissue (B220-TLT; dot circle) (**Figure 18 D**).

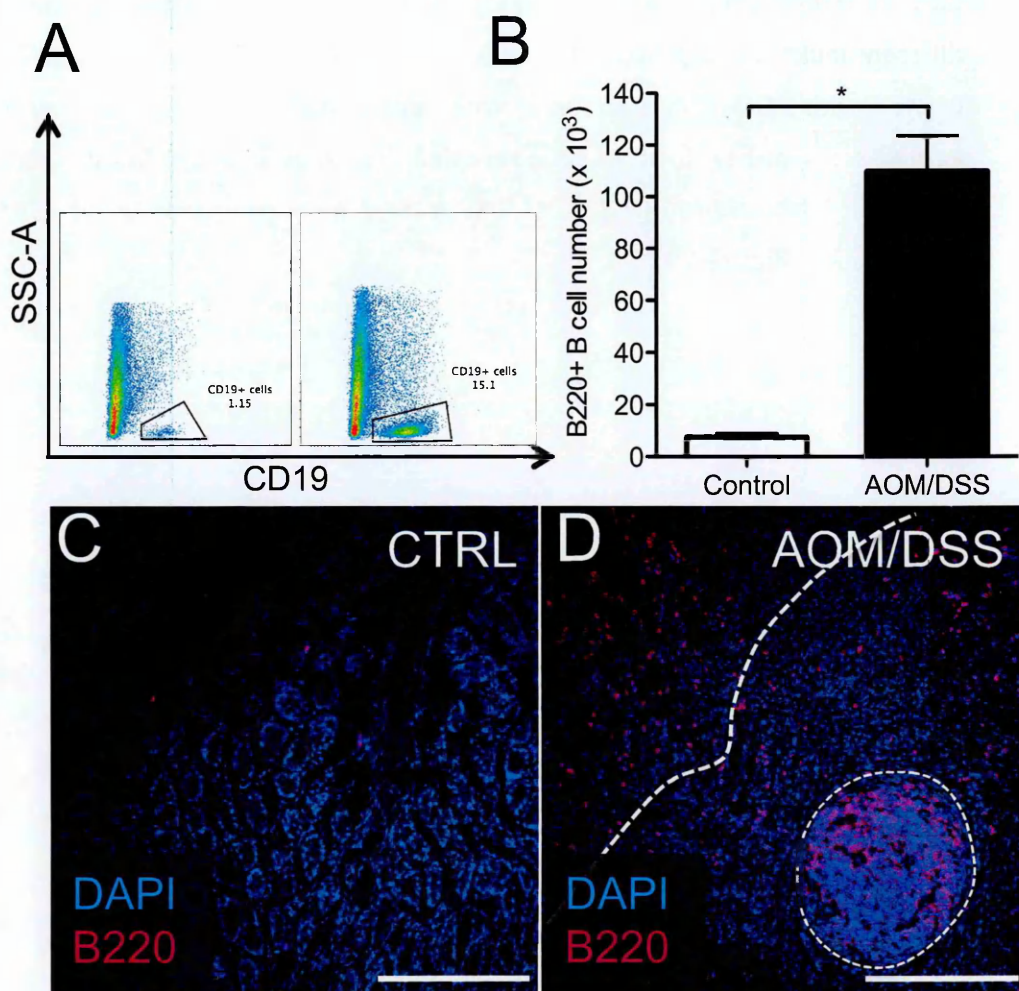


Figure 18. B cell analysis in a preclinical model of colorectal cancer.

B cell number increases in AOM/DSS treated mice compared to controls, as summarised in dot plots (**A**), which represent respectively B cells from controls and tumour-bearing mice, and in the histogram (**B**). Histograms represent mean of three experiments ($n = 6$ mice, Ctrl; $n = 12$ mice AOM/DSS; bars represent SEM). * $p < 0.05$ by Student's t test. Frozen sections of murine colon from control mice (**C**) and AOM/DSS mice (**D**) stained with anti-B220 antibody (red). B cells showed a dual localization: they are a major component of TLT (**dotted circle, D**) and they also localize interspersed in the tumour tissue (**dotted line, D**). Scale bars: $100 \mu\text{m}$. * $p < 0.05$ by Student's t test.

Once established that B cell number increases in AOM/DSS treated mice compared to controls, I wondered how B cells affect other leukocyte populations infiltrating CRC. To address this question, I analysed by flow cytometry the distribution of different leukocyte populations in wt mice and in mice lacking B cells (μ MT), both treated with AOM/DSS. A staining with several antibodies (anti-CD3, anti-CD8, anti-NK1.1, anti-F4/80, anti-Ly6G) revealed that there is a significant increase in leukocyte infiltration in μ MT AOM/DSS treated mice compared to wt AOM/DSS treated mice (**Figure 19**).

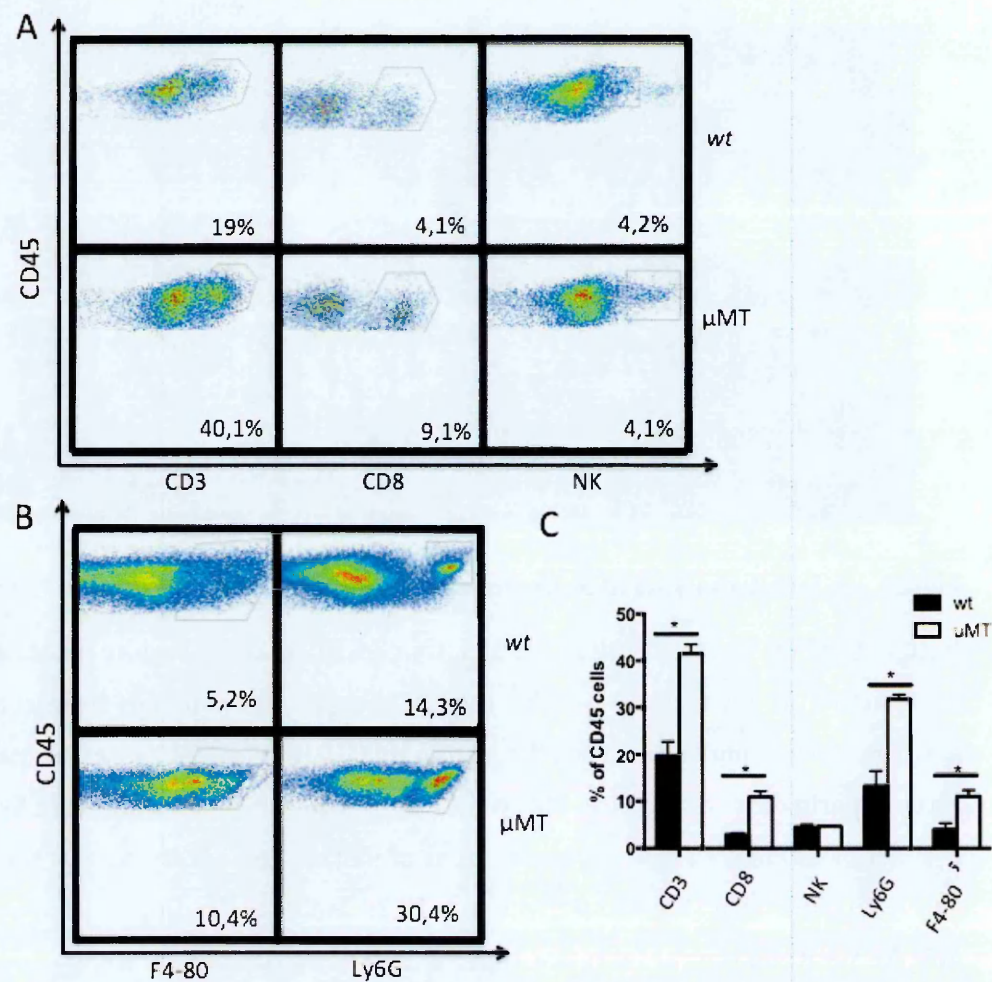


Figure 19. Analysis of the immune infiltrate in wt vs μ MT AOM/DSS treated mice. The recruitment of immune cell populations increases in μ MT AOM/DSS treated mice, as summarised in dot plots (A-B). There is a significant increase of CD3+ T cells and CD8+ T cells (A-C) and of macrophages and neutrophils (B-C). * $p < 0.05$ by Student's t test

In the previous chapter, I have shown that the treatment of wt mice with AOM/DSS induces the expansion of lymphoid tissue associated to the colon mucosa, with B cells representing the major population [53]. Due to the absence of B cells in μ MT mice, in order to visualize lymphoid aggregates, I have stained μ MT colon whole mounts with Syto green, a specific dye for nuclei. Surprisingly, while B220-TILs are absent in the colonic mucosa of μ MT/AOM/DSS mice (**Figure 20A**), nuclear staining evidenced aggregates of cells resembling TLT (**Figure 20A**), thus suggesting that B cells are not dispensable for TLT formation. TLT in the colon mucosa of μ MT/AOM/DSS mice included a dense core of CD11c dendritic cells (**Figure 20B**), scattered CD3+ T cells (**Figure 20C**), PNA⁺ high endothelial venules (HEV) (**Figure 20D**), Lyve1+ lymphatic vessels and the lymphorganogenic chemokine (CCL21) (**Figure 20E**) suggesting that, although devoid of B cells, TLT is fully equipped to mediate T cell recruitment, which represents its important function.

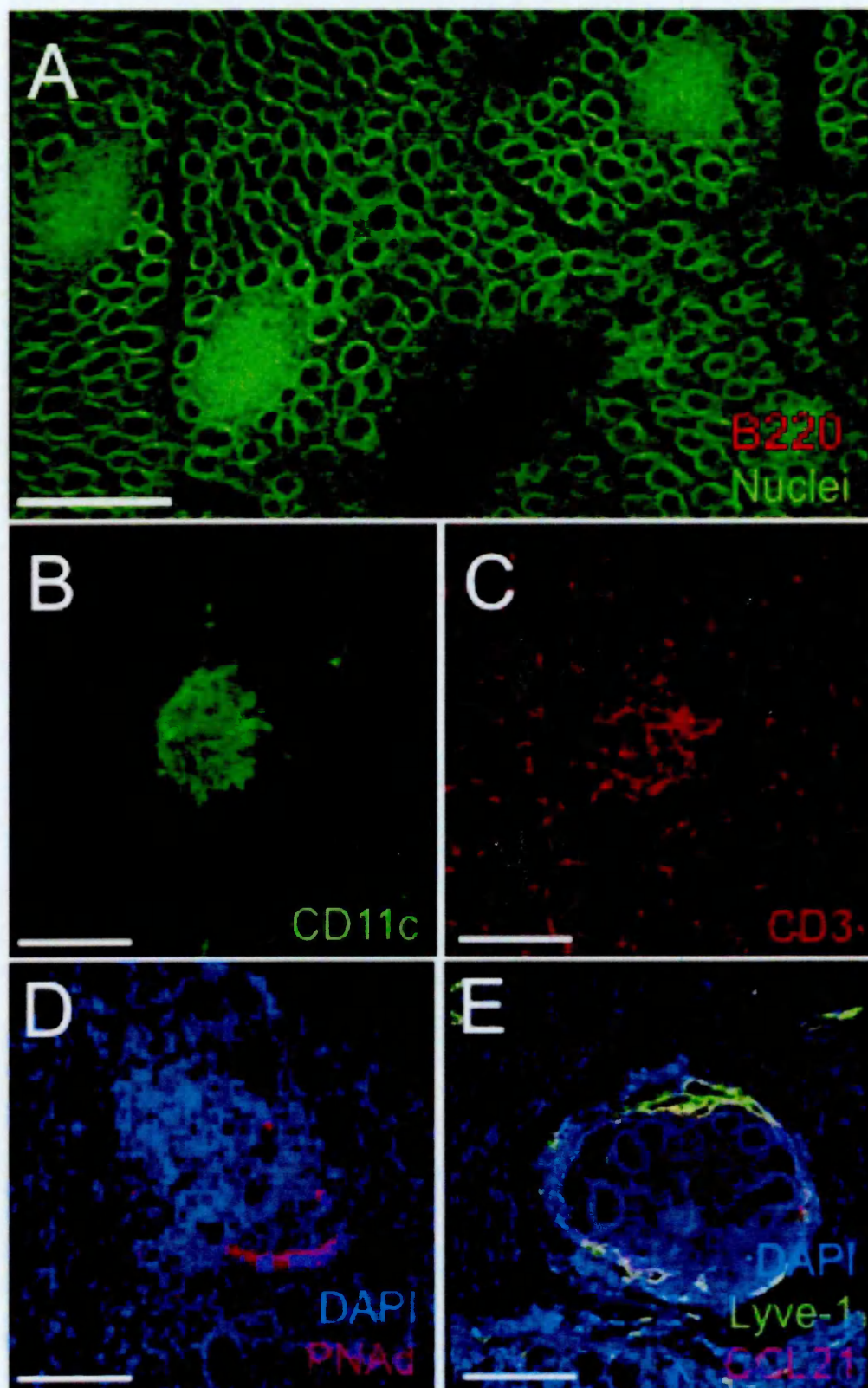


Figure 20. Characterization of TLT in μ MT AOM/DSS treated mice. Colon whole mounts have been stained with syto green (Green in A), anti-B220 (red in A), anti-CD11c (B), anti-CD3 (C). Frozen sections have been stained with anti-PNAd (D), anti-Lyve1 (green in E) and anti-CCL21 (red in E) antibodies. TLT is present even if its major component (i.e. B cells) is absent (A). The majority of cells are CD11c+ dendritic cells (B) PNAd+ HEVs (D) and Lyve+ lymphatics (green, E). The lymphorganogenic chemokine CCL21 is also present and associated to lymphatic endothelium (red, E). Scale bars: 100 μ m (A-C), 50 μ m (D-E).

Quantification of TLT confirms that, despite B cell deficiency, TLT expands during carcinogenesis, although to a lesser extent than in wt mice (Figure 21).

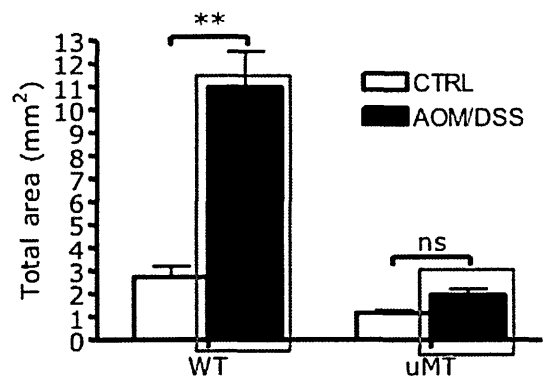


Figure 21. Quantification of TLT in μ MT /AOM/DSS mice. Colon whole mounts have been stained with Syto 13 green, in order to visualize cell nuclei, thus allowing the identification of lymphoid tissue, which appears as a dense aggregate of nuclei. TLT density has been evaluated by morphometric analysis on colon whole mounts. There is a slight but not significant expansion of TLT in μ MT /AOM/DSS mice compared to μ MT controls, ** $p < 0.01$ by Student's t test

To investigate the contribution of B cells in the colorectal cancer microenvironment, I evaluated tumour growth in B cell-genetically deficient mice (μ MT). Ablation of B cells significantly increased the number of adenomas in the colorectal mucosa (**Figure 22**). Considering the prevalent distribution of B cells within TLT in the colon mucosa of AOM/DSS mice (**Figure 18D**), this suggests that B cells within TLT might exert an important antitumour function. These data are in accordance with our human data and show that B cells within TLT seem to restrain tumour growth.

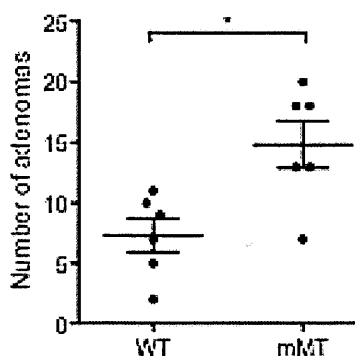


Figure 22. Evaluation of tumour growth in the μ MT/AOM/DSS model. Quantification of adenomas in the AOM/DSS preclinical model. AOM/DSS treated μ MT mice show a higher number of polyps compared to AOM/DSS treated wt mice. n= 6 wt, n= 6 μ MT; * p < 0,05 by Student's t test

The antitumour role of B cells within TLT could be related to a variety of B-cell functions, including the capability of TLT to sustain a germinal centre reaction. Since I have already shown that B cells in the human colorectal mucosa are predominantly IgG+, both dispersed and localized within tumour-associated TLT, I also analysed the Ig repertoire expressed by B cells in the CRC preclinical model. I quantified the concentration of immunoglobulins by an ELISA assay performed on supernatants recovered from mouse stool, representative of the production of Ig in the colonic mucosa. I have quantified IgA, IgM, IgG1, IgG2b, IgG3 with specific antibodies. Results showed that IgA, the most prevalent antibody at mucosal sites,

decrease in AOM/DSS mice compared to controls (**Figure 23 A**), while the other immunoglobulins increase (**Figure 23 B-E**). This indicates a skewed immunoglobulin repertoire during carcinogenesis and suggests that the presence of TLT associates to B cell maturation and isotype switch and the generation of a humoral immune response, possibly accounting for an antitumor function.

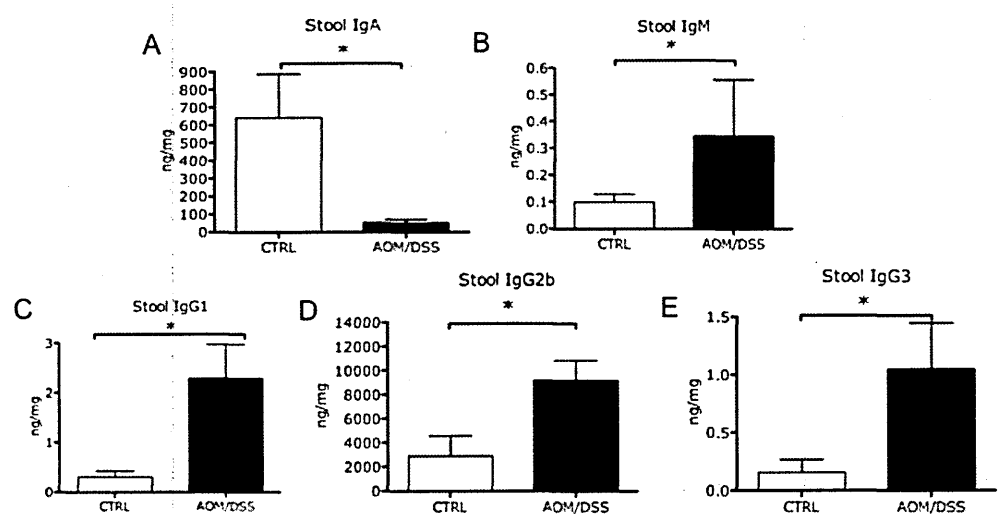


Figure 23. Analysis of immunoglobulins in the stool of AOM/DSS mice. Immunoglobulins have been quantified by ELISA using specific antibodies: anti-IgA, anti-IgM, anti-IgG1, anti-IgG2b, anti-IgG3. Stool IgA are more abundant in control mice compared to tumour-bearing mice (**A**), while IgM, IgG1, IgG2b, IgG3 increase in AOM/DSS mice compared to controls (**B-E**). * $p < 0.05$ by Student's T test

Evaluation of tumour growth and analysis of the immune infiltrate in the CMT93 model

In the second preclinical model, I have subcutaneously injected both wt and μ MT mice with a murine cell line of CRC, the CMT93 cell line. In contrast to the AOM/DSS model, B cells only distributed scattered in the tissue, while in none of the tumours analysed I could document occurrence of TLT (not shown), likely due to the fast growth of the tumour mass, compared to the AOM/DSS model. Consistent with the distinct pattern of B cell distribution observed, genetic B-cell deficiency oppositely affected tumour growth in the CMT93 preclinical model compared to the AOM/DSS. Both tumour incidence (**Figure 24A**) and growth (**Figure 24B**) of CMT93 cells was reduced in μ MT mice compared to controls, thus suggesting that interspersed B220-TILs might have a pro-tumoural role.

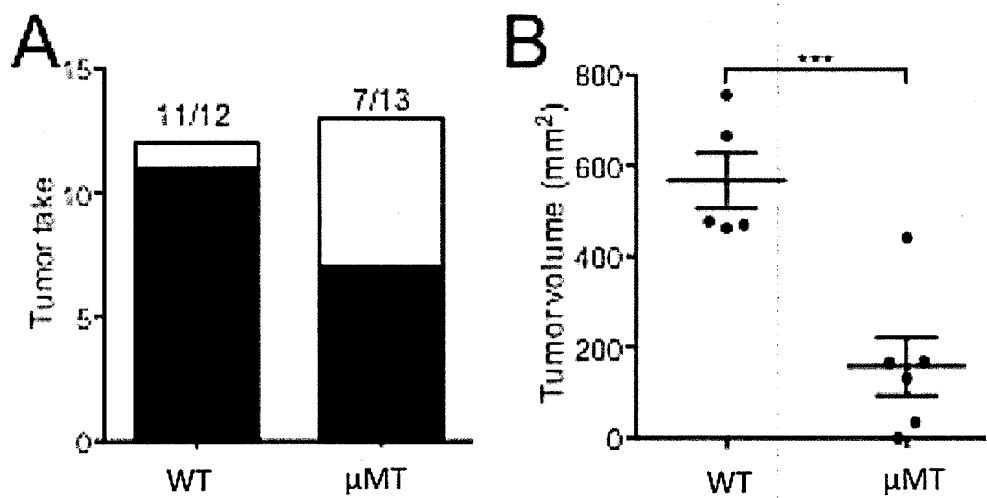


Figure 24. Evaluation of tumour growth in the CMT93 model. Quantification of tumour incidence and volume in the CMT93 preclinical model of CRC. Tumour incidence (A, $n = 12$ wt, $n = 13$ μ MT) and volume (B, $n = 5$ wt, $n = 6$ μ MT) is reduced in μ MT mice compared to controls. $p = 0.03$ by Chi square (A); *** $p < 0.001$ by Student's T test (B)

Thus, consistent with our hypothesis of a different role of B cells depending on their localization in the tumour tissue (here modelled by the two different preclinical models), and also in accordance with our clinical data, B cells seem to sustain tumour growth in colorectal cancer, when infiltrating the microenvironment as interspersed B-TILs. To investigate how B cells could contribute to a protumour function, I asked whether they modulate the type of immune response. To this aim, I performed a characterization of the immune infiltrate in the CMT93 model. Flow cytometric analysis of whole colons from wild type and μ MT CMT93 tumours indicated a significant and selective increased infiltration of T lymphocytes, including CD8+ T cells as compared to tumours growing in wild-type mice (**Figure 25**).

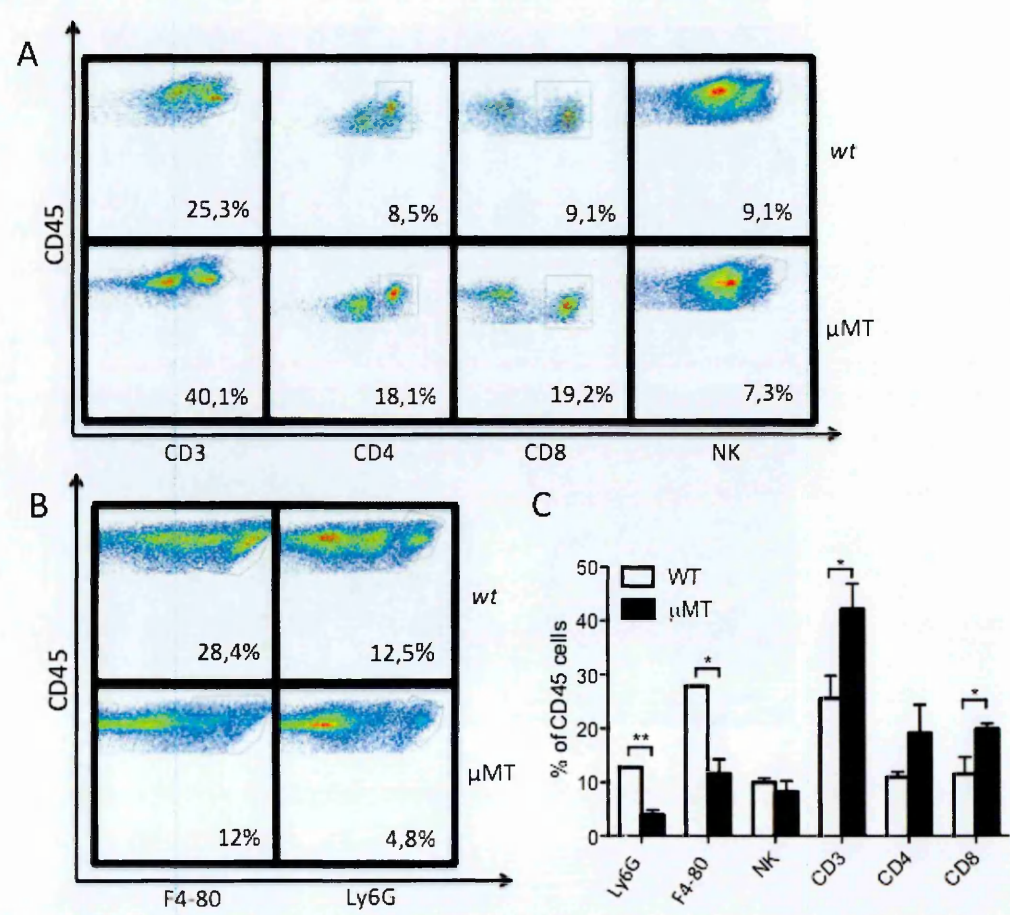


Figure 25. Analysis of the immune infiltrate in wt vs μ MT CMT93-injected mice. There is a significant increase of T lymphocytes, including CD3 and CD8 + cells, in μ MT mice compared to wt mice as shown in dot plots (A) and summarised in the histograms (wt: white bars; μ MT: black bars; C). Panel B shows the infiltration of macrophages and neutrophils, which is significantly higher in wt mice compared to μ MT mice (B), as summarised in panel C (C). * $p < 0.05$; ** $p < 0.01$ by Student's T test

I then performed immunofluorescence stainings with anti-CD3 and anti-CD8 antibodies on tumour sections and analysed samples by confocal microscope, confirming that there is an increase in tumour infiltrating CD3+T cells and CD8+T cells in mice lacking B cells (**Figure 26 C-D**) compared to tumour-bearing wt mice (**Figure 26 A-B**).

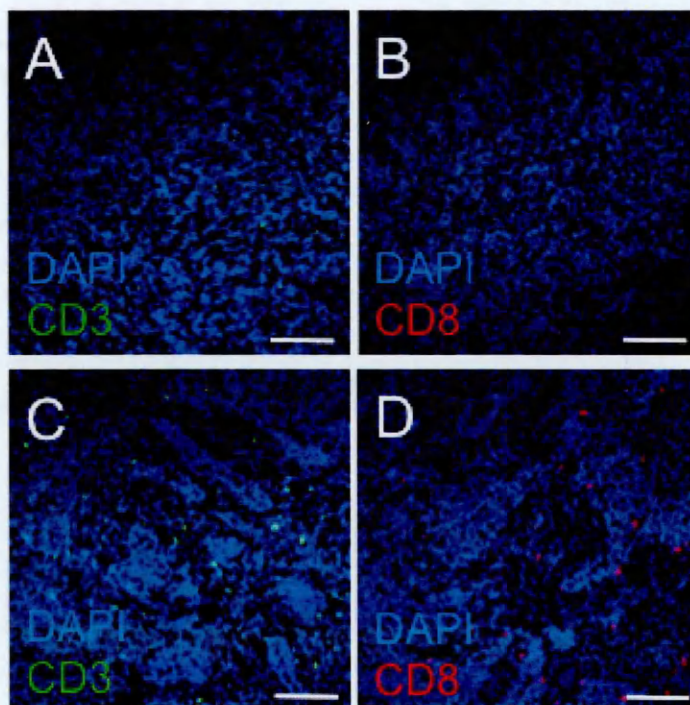


Figure 26. Analysis of the immune infiltrate in CMT93 tumours. Frozen sections of CMT93 subcutaneous tumours in wt (upper panels) and μ MT (lower panels) mice were stained with anti-CD3 (green, A-C) and anti-CD8 (red, B-D) antibodies. Tumours from mice lacking B cells have an increased infiltrate of immune cells, specifically CD3+T cells and CD8+T cells compared to wt tumour-bearing mice. Scale bars: 50 μ m

Finally, I further analysed the type of immune response by evaluating the gene expression profile of CMT93 tumours. In particular, I tested genes related to the anti-tumour immune response, such as Granzyme, Perforin, TNF. I found that activated several genes, including IL-12, Granzyme A and B, Perforin, INF-gamma and TNF-alpha are highly expressed in μ MT tumour-bearing mice compared to controls (**Figure 27**). These results suggest that, in an implanted tumour model devoid of B220-TLT, depletion of tumour infiltrating B cells (B-TILs) increase the recruitment of important components of the antitumour immune response.

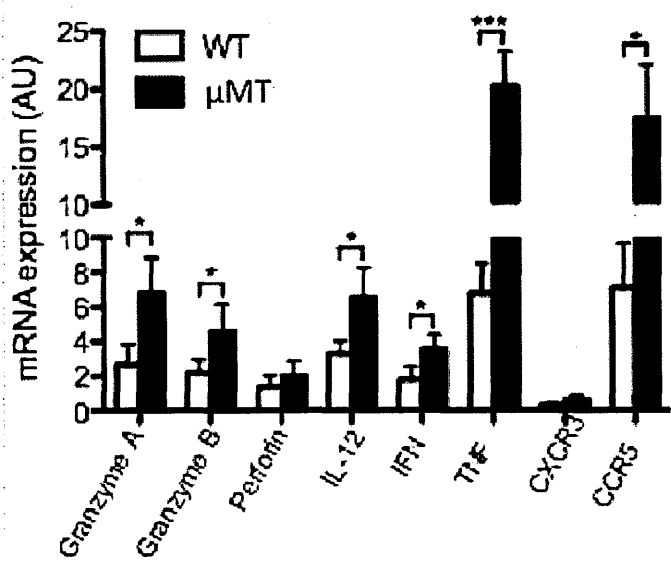


Figure 27. Gene expression profile in CMT93 tumours. Gene expression performed on RNA from CMT93-tumours, from both wt and μ MT mice. There is a strong up-regulation of the genes related to the anti-tumour immune response in μ MT mice (black columns) compared to wt mice (white columns). Granzyme A, Granzyme B, IL-12, INF, TNF, CCR5 are significantly up regulated. * $p < 0.05$, *** $p < 0.001$ by Student's t test.

Preliminary analysis of CD20-TILs in human CRC

According to the data shown so far, B cells have a different influence on tumour growth, depending on their localization within the colon mucosa during tumorigenesis. My analysis of B-cell function in different preclinical models suggests a potential antitumour function for B cells when localized within TLT and a pro-tumour function of diffusely infiltrating B cells. As a preliminary explanation for this duality, I have provided evidence that B cells regulate the type of immune reaction in CRC, as their absence is associated to increased infiltration of important components of the antitumour immune response (**Figure 25-27**). Besides their primary role as antibody-producing cells, B cells can also release modulatory cytokines with important regulatory function [72]. IL-10-producing B cells have been recently implied in the restrain of excessive inflammation [163] and in cancer [76]. In addition, a population of PDL-1 expressing B cells has been described to be involved in immunosuppression in prostate cancer [73]. Thus, based on this literature and in order to preliminary investigate the mechanisms underpinning B-cell pro-tumour function in CRC, I focused on two key molecules with immunosuppressive function, IL-10 and PD-L1. By immunohistochemistry with specific antibodies against IL-10 and PD-L1 on human colorectal cancer specimens, I identified scattered CD20+ B cells expressing IL-10 (**Figure 28 A**), which, in contrast, was not expressed by B cells within TLT (**Figure 28 B**) and few CD20-TILs showing PD-L1 expression (**Figure 28 C**).

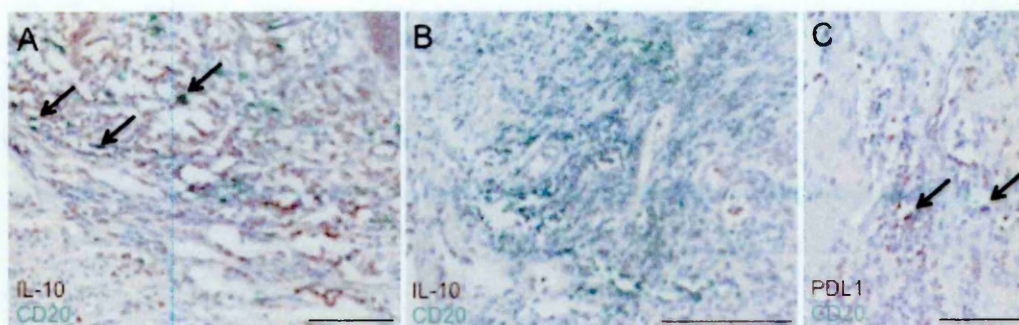


Figure 28. CD20-TIL expression of immunosuppressive cytokines in human CRC. Expression of regulatory molecules by CD20-TILs. Immunohistochemistry with anti-IL-10 and CD20 (A-B). Some CD20-TILs show positivity for both IL-10 (A), which is not expressed in B cells within TLT (B) and PD-L1 (C). Scale bars: 100 μ m.

Discussion

In the second part of my thesis, I have presented data about the involvement of B cells in tumour progression in the microenvironment of CRC. Surprisingly, while T cell subsets occupy a prime position in studies aimed at assessing the role of leukocytes in cancer, cells of the humoral arm of the immune system have shown an unconvincing behaviour in most prognostic studies. Nonetheless, B and T cells are often localized in tight association into aggregates in the tumour microenvironment, possibly reflecting how they interact during the organization of a local immune response [92, 143]. In the first part of the results, focused on T cells, I showed that B cells represent a relevant component of TLT, both in humans and in mice. However, as for T cells, visualization of B cells in the microenvironment by immunohistochemistry revealed that B cells distribute in distinct regions at the tumour-host interface: they localised both in TLT in the stromal compartment (CD20-TLT) and scattered at the tumour margin (CD20-TILs). Despite whole tissue visualisation of CD20+B cells evidenced that they were preferentially located within TLT, instead of being diffuse within the tissue as TILs, the peculiar distribution in two distinct histo-pathological compartments prompted me to evaluate the role of B cells according to their localization. I found that CD20-TLT associated to better prognosis, while CD20-TILs did not. Interestingly, CD20-TLT coordinated with CD20-TILs only among patients who experienced cancer recurrence. This results suggests that, when located within a lymphoid site, B cells might be involved in an ongoing follicular anti-tumour immune response with a protective anti-tumour role. Conversely, the distribution of B cells scattered in the microenvironment is likely to reflect a non-specific pro-tumour inflammatory reaction.

To partially explain the antitumour function of B cells within TLT, I explored whether TLT could be site for immune response, by analysing the immunoglobulin profile and two proliferation markers, representative of an active GC. Among tumour infiltrating B cells, very few or none were of the IgA subclass, recently

associated to an immunosuppressive function in prostate cancer [73], while predominantly of the IgG subclass, suggesting a different antigenic response compared to normal colonic mucosa. Moreover, within TLT, I observed germinal centres, characterized by Ki-67+ proliferating cells and BCL6+ cells, suggesting that TLT have the potential to sustain switch recombination and somatic hyper mutation, which are required for the generation of an effector and memory B cell response. Overall, the profile of the B cell compartment of TLT suggests that this immune site is fully equipped to sustain B cell antibody production in human CRC, which can be crucial trigger of anticancer cellular immune responses [161].

I then took advantage of different CRC preclinical models, in order to dissect the role of the distinct B cell components (B-TILs and B-TLT).

What I found is that, in a model in which B cells localize primarily within TLT and also spread in the tissue, the absence of B cells significantly increased tumour formation, suggesting that B cells within TLT might exert an important anti-tumour function, which, according to my previous data, could be related to the capability of TLT to sustain a GC reaction. In contrast, in a model in which B cells only localize within the tissue, genetic ablation of B cells reduces tumour growth, suggesting that infiltrating B220-TILs might have a pro-tumour role, which could be related, according to my results, to inhibition of important components of the anti-tumour immune response. B-TILs could have a non-protective role, likely to be mediated by the production of immunosuppressive molecules, including IL-10 and PDL-1. IL-10 positive B cells are B regulatory cells that contribute to the inhibition of excessive inflammation and support the immunological tolerance [72]. B reg cells are involved in the control of inflammation by IL-10 production in preclinical models of colitis [164], in EAE (Experimental Autoimmune Encephalomyelitis [83] and arthritis [165]. PD-L1 is expressed on many cancer and immune cells and plays an important role in blocking the anti-tumour immune response by binding PD-1, negative regulator of T-lymphocyte activation; it reduces cytokine production and suppresses T cell proliferation [166]. Its expression on B cells could be important immunosuppressive signal when B cells are engaged in antigen-presenting functions.

One interesting point that has emerged from my thesis is the importance of immuno-histo-pathological methodologies, carefully evaluating immune cells in the microenvironment. Gene-expression-based and FACS-based analyses are the most commonly used methods to address the prognostic role of immune cell populations. In the context of CRC, the localization and the structural organization of immune cells are key regulators of their function, as evidenced by my results. Overall, a new idea has emerged: the relevance of B cells in human cancer should take into account their localization with regard to lymphoid neo-genesis, which plays an important role in dictating their function in the tumour microenvironment.

The role of B cells in the progression of solid tumours has fostered studies concerning their targeting by anti-CD20 antibodies [162, 167-169]. In relation to the role I have found for CD20-TLT and CD20-TILs, respectively anti-tumour and pro-tumour, in the context of immunotherapy, I can speculate that targeting pro-tumour CD20-TILs could be an effective immunotherapeutic tool against tumour progression, whereas depletion of CD20-TLT with anti-tumour properties might be counterintuitive in the clinical context of CRC. Thus, the effectiveness of anti-CD20 treatment in solid tumours might be the result of its ability to selectively target interspersed CD20-TILs, while being less efficient in depleting B cells within TLT at the tumour margin. A considerable amount of studies showed that genetic inactivation of B cells induced tumour growth and unleashed adaptive immune cells [170, 171], while a previous study in an experimental tumour model argued against B cell depletion showing a higher tumour growth and impaired CD4+ and CD8+ T cell induction after anti-CD20 treatment [172]. Discrepancies between studies were explained by claiming developmental differences in the immune repertoire between genetic and late depletion of B cells. Thus, the effectiveness of anti-CD20 treatment in solid tumours that has been previously observed in both clinical [168] and experimental models [168] might be the result of its ability to selectively target interspersed CD20-TILs, while being less efficient in depleting B cells within TLT at the tumour margin. Design of novel immunotherapeutic drugs depleting B cells should take into account their ability to selectively targeting CD20 TILs but not CD20-TLT. In this regard, the kinetic of B cell depletion by anti-CD20

antibodies varies among different B cell sub-populations (i.e. circulating, marginal zone, peritoneal) [173], thus suggesting that it's possible to preferentially target specific subsets. Moreover, in non-tumour conditions, anti-CD20 therapy has resulted ineffective in depleting CD20⁺ cells in tertiary lymphoid organs [174], suggesting that B cells might receive survival signals within lymphoid niches [173]. The differential sensitivity that B cells display to anti-CD20 treatment according to their localization could be exploited to design tailored approaches, assuming that a rigorous evaluation of B cell spatial distribution is performed.

CONCLUDING
REMARKS
and
THERAPEUTIC
IMPLICATIONS

In my thesis I have examined the occurrence of TLT in the microenvironment of human CRC, the mechanisms driving its formation, its capability to mediate T cell recruitment and its relevance as a prognostic immune variable. In a second part, I have moved forward to examine the function of the humoral component of TLT, i.e. B cells, and explored the duality of these immune cells in relation to their spatial distribution in the microenvironment of human CRC. My data, overall, suggest that tertiary lymphoid tissue is a relatively novel component of the immune microenvironment of colorectal cancer with a key role as prognostic tool and in the design of novel immunotherapeutic strategies.

The tumour microenvironment is the site where host and cancer cells interact. Within the tumour microenvironment, tumour complexity is due not only to the intrinsic heterogeneity of cancer cells, but also to the variety of tumour infiltrating immune cells. The anti-tumour function of adaptive immune cells is target of immunotherapeutic strategies, aiming at stimulate the anti-tumour immune response. At the same time, immunosuppressive circuits are targets of therapeutic approaches aimed at reverting the exhausted immune landscape and boosting an effective immune response. Research in tumour immunology is thus strengthening the knowledge of the mechanisms driving the action of immune cells in the tumour context, with the ultimate goal to provide new diagnostic, therapeutic and prognostic tools to the clinical practice [33, 87]. However, the clinical characterization of tumour infiltrating cells needs to be ameliorated, in order to identify ideal targets of effective therapeutic strategies.

In the last decades, tumour profiles generated by standardized wide-scale analyses have highlighted a central position of immune variables in dictating tumour progression [93, 175], suggesting that specific immune subsets should be included within the currently available TNM staging system in the determination of patient's prognosis [61]. Thus, cancer research is constantly engaged in the identification of the specific immune profile of each solid tumour. Overall, these studies highlight that effector T cells in the tumour environment correlate with a better tumour prognosis [35, 94, 176]. Inflammatory mediators are undoubtedly

involved in CRC initiation as well as progression. However, CRC is a tumour where most consistently tumour-infiltrating T cells have been demonstrated to have a protective role, being significantly associated with better clinical outcome [60, 61].

Despite the presence of effector T cells in solid tumours correlates with a favourable prognosis in the majority of tumour types, the mechanisms of T cell recruitment and activation largely remain to be defined. In a physiologic context, immune responses are generated within lymphoid organs thanks to specific cellular and molecular interactions. Anti-tumour T cells get activated in SLO, after tumour-antigen retrieval by DCs in the inflamed microenvironment [177, 178]. Once activated, T cells migrate to the tumour, where they exert their anti-tumour function against tumour-antigen expressing cells. Lately, it has been shown that, occasionally, adaptive immune responses and priming of naïve T cells could arise in ectopic lymphoid sites in peripheral tissues. TLT has been well characterized as an ectopic immunological compartment with a highly organized structure. It mediates lymphocyte recruitment from the blood and it could give a contribution to the generation of adaptive immune responses [92, 145, 176], thanks to topological compartmentalization, the presence of stromal cells and a specialized vessel network.

It is now established that TILs have a positive prognostic role in solid tumours, reflecting the potential ability of adaptive immune cells to exert tumour control. Recently, the approach to investigate the relevance of TILs in human cancer has been improved: the analysis of adaptive cells is combined with the definition of specific subsets and their quantitative distribution in the tumour microenvironment. Despite the advent of innovative prognostic tools, such as global molecular analyses, histopathological examination of immune cells in tumours still stands as one of the most powerful approaches to assess the relevance of the complex immune microenvironment. It's only thanks to careful histopathological examination of immune variables within the microenvironment that it has been possible to appreciate the spatial organization of immune cells as

an important determinant of immune cell function and a critical feature in establishing patient outcome [52, 144].

In 1967, pathologists, analysing haematoxylin and eosin (H&E)- stained tumour tissue slides, found that an unspecific lymphocytic “inflammatory reaction” could represent a prognostic feature, although devoid of information on specific subpopulations of immune cells [179]. Now a day, it is possible to create a reproducible immune signature in definite tumour locations, for example at the invasive margin, thanks to both the development of immunohistochemistry-based methodologies for the detection of specific lymphocyte surface markers and computer-based quantitative image analysis. This binomial makes possible an accurate assessment of the prognostic role of TIL subsets

Cancer immunotherapy is known as a group of strategies which their purpose is to activate immune mechanisms or neutralise inhibitory and suppressing immune networks. Among innovative immunotherapeutic strategies, those aiming at increase the extent of TILs in tumour microenvironment are supported by the prognostic relevance of TILs. Recently, thanks to preclinical and clinical studies, TLT has become a critical player in this model, since it sustains the recruitment and activation of immune cells to the tumour microenvironment. One of the limit in the success of immunotherapeutic approaches is the homing of anti-tumour T cells to the tumour. In this scenario, the development of TLT in the tumour microenvironment could represent an useful instrument that facilitates the recruitment of effector T cells in those areas barely accessible. Moreover, the detection of TLT at the tumour site during immunotherapy might be a monitoring strategy useful in identifying patients who are prone to respond to immunotherapy strategies aimed at boosting the host immune response. Lutz et al demonstrated that TLT was induced in 33 out of 39 pancreatic cancer patients enrolled in a clinical trial [180]. The core of the study consisted in an irradiated granulocyte colony stimulating factor (GM-CSF)-secreting allogeneic pancreatic cancer vaccine (GVAX). With this trial, it has been demonstrated that TLT can be

targeted by immunotherapy to orchestrate T cell infiltration and participate in the generation of an immunogenic environment [180].

Due to their ability to activate lymphocytes, DCs might represent a central actor in immunotherapy. Accordingly, it has been shown that activated DCs can sustain the formation of organized lymphoid tissue in preclinical models [181, 182]. Data reported are supported by the presence of DCs in lymphoid organs and their pivotal role in the initial aggregation of lymphocytes during lymphorganogenesis and in vessel formation. However, most of clinical trials with DC-based immunotherapy stopped at phase II.

B and T cells are often localized in tight association into aggregates in the tumour microenvironment, possibly reflecting how they interact during the organization of a local immune response [54, 92, 152, 183]. In my study, I have documented that B cells in human CRC localized both in tertiary lymphoid tissue in the stromal compartment (CD20-TLT) and scattered around the tumour margin (CD20-TILs). B cells within tertiary lymphoid structures were shown to be antigen specific and to correlate to favourable prognosis in NSCLC [54]. In our analysis, CD20-TLT associated to better prognosis, suggesting that, when localized within a lymphoid site, B cells might be involved in an ongoing follicular antitumor immune response with a protective antitumor role. Differently, B cells scattered in the tumour tissue were not associated with better prognosis and notably CD20-TLT coordinated with B cells only among patients who experienced cancer recurrence. Thus, it is tentative to speculate that while the recruitment of scattered B cells is likely to reflect a non-specific pro-tumour inflammatory reaction, the presence of B cells as a component of lymphoid aggregates mirrors an antigen-driven expansion with a potential protective antitumor role. Our analysis highlights the importance of B cell distribution in predicting colorectal cancer prognosis and raises criticism about studies addressing the prognostic role of B cell signatures obtained by gene expression-based or FACS-based analyses, due to the unspecific quantification of B cells with regard to their localization in the tumour microenvironment [184] and highlight the importance of immunohistopathological methodologies in this

context. Our study brought to light the idea that the relevance of B cells in human cancer should take into account of their localization and geographical distribution with regard to lymphoid neogenesis, which plays an important role in dictating their function in the tumour microenvironment.

In the context of novel immunotherapy treatments, our data propose that targeting pro-tumour CD20-TILs could be an effective immunotherapeutic tool against tumour progression, whereas depletion of CD20-TLT with anti-tumour properties might be counterintuitive in the clinical context of CRC. Our study suggest that novel immunotherapies strategies aimed at depleting B cells in solid tumours [185] should be tested for their anti-tumour properties in experimental models according to their ability to generate the de-novo formation of TLT in the tumour microenvironment.

The design of novel immunotherapeutic strategies able to increase lymphocyte recruitment and activation at the tumour site is supported by the prognostic abilities of TILs. In this context, a new central actor has been proposed in the classification of patient survival: the presence of TLT in the tumour microenvironment. If TLT is an active and functional immune site that participate in the recruitment and activation of T cells in the tumour microenvironment, as experimental and clinical studies report, then immunotherapeutic approaches could exploit the occurrence of TLT to increase the delivery of effective T cells to otherwise unreachable tumour areas.

LIST OF ABBREVIATIONS

CRC	Colorectal cancer
TIL	Tumour infiltrating lymphocytes
TLT	Tertiary lymphoid tissue
HEV	High endothelial venules
GFP	Green fluorescent protein
WHO	World health organisation
IBD	Inflammatory bowel disease
HNCCP	Hereditary non polyposis colorectal cancer
FAP	Familiar adenomatous polyposis
MLH1	MutL homolog 1
MSH2	MutS homolog 2
APC	Adenomatous Polyposis Coli
CIN	Chromosomal instability
MSI	Microsatellite instability
TNM	Tumour Node Metastasis
EGFR	Epidermal growth factor receptor
MMR	Mismatch repair
mAbs	Monoclonal antibodies
VEGF	Vascular endothelial growth factor
PD1	Programmed cell death-1
PDL1	Programmed cell death-1 ligand
CAC	Colitis-associated colorectal cancer
MDSC	Myeloid-derived suppressor cells
NK	Natural killer
DC	Dendritic cells
VELIPI	Vascular emboli, lymphatic invasion and perineural invasion
Th1	T helper 1

Breg	B regulatory cell
IL-10	Interleukin 10
TGF-β	Transforming growth factor beta
APC	Antigen-presenting cells
CT	Centre of the tumour
IM	Invasive margin
DFS	Disease free survival
DSS	Disease specific survival
OS	Overall survival
MALT	Mucosal associated lymphoid tissue
GALT	Gut-associated lymphoid tissue
IEL	Intra-epithelial lymphocytes
LPL	Lamina propria leukocytes
PP	Peyer's Patch
IgA	Immunoglobulin A
IgG	Immunoglobulin G
MLN	Mesenteric Lymph Nodes
CP	Criptopatch
ILF	Isolated lymphoid follicles
LTi	Lymphoid tissue inducer
SCF-R	Stem cell factor receptor
IL-7R	Interleukin 7 receptor
CSR	Class Switching Recombination
SLO	Secondary lymphoid organ
GC	Germinal Centre
ELS	Ectopic lymphoid-like structures
FRCs	Follicular reticular cells
FDC	Follicular dendritic cells

TNF	Tumour necrosis factor
LTo	Lymphoid tissue organizer
LTi	Lymphoid tissue inducer
LTβR	Lymphotoxin beta receptor
LTα₁β₂	Lymphotoxin alpha 1 beta 2
ACPA	Anti-citrullinated protein antibodies
HP	Helicobacter pylori
IDC	Infiltrating ductal carcinoma
TI-BALT	Tumour Induced – Bronchus-Associated Lymphoid Tissue
NSCLC	Non-small-cell lung cancer (NSCLC)
AOM	Azoxymethane
DSS	Dextran sulphate sodium
ApcMin	Adenomatous Polyposis Coli - Multiple intestinal neoplasia
IRA	Immune reactive area

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